UNIVERSITY OF ALBERTA

NOVEL INTERVENTIONS IN THE HEART AND PLATELETS: FROM BENCH TO BEDSIDE

BY

PO-YIN CHEUNG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF PHARMACOLOGY

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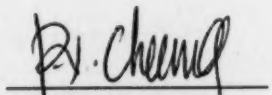

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **NOVEL INTERVENTIONS IN THE HEART AND PLATELETS: FROM BENCH TO BEDSIDE** submitted by **PO-YIN CHEUNG** in partial fulfillment of the requirements of the degree of **DOCTOR OF PHILOSOPHY.**

R. Schulz (Supervisor)

T. Gordon (Committee Member)

S. E. Jacobs-Kaufman (Committee Member)

M. W. Radomski (Committee Member)

C. R. Triggle (Attending External Examiner)

A. S. Clanachan (Committee Chair & Examiner)

DATE: Sept. 8, 1998

ABSTRACT

Nitric oxide is a ubiquitous molecule that regulates blood flow and platelet function. However, during myocardial ischemia-reperfusion, nitric oxide can be cytotoxic due in part to the formation of peroxynitrite by reacting with superoxide. Glutathione is an important antioxidant in cells and can be a unique scavenger of peroxynitrite with the formation of a nitric oxide donating intermediate such as *S*-nitrosoglutathione that can stimulate soluble guanylate cyclase. I found that exogenous administration of glutathione caused concentration-dependent coronary vasodilation in isolated rat hearts via a nitric oxide- and cyclic GMP dependent mechanism. I also found that exogenous glutathione alleviated myocardial reperfusion injury in isolated rat hearts, which was associated with decreased peroxynitrite release into the coronary circulation and elevated myocardial cyclic GMP levels. Furthermore, in heart homogenates, glutathione and thiols that contain a free sulfhydryl group protected myocardial aconitase from inhibition by peroxynitrite.

In addition to its role in ventricular remodeling following myocardial infarction, matrix metalloproteinase-2 is a novel mediator of platelet aggregation. Using gelatin embedded zymography, I demonstrated that matrix metalloproteinase-2 was released into the coronary circulation from the heart and the release was acutely elevated during reperfusion following global, no-flow ischemia. Inhibition of matrix metalloproteinase-2 with its antibody or by the zinc chelating agent *o*-phenanthroline improved cardiac mechanical function during reperfusion.

During extracorporeal membrane oxygenation in neonates, a novel

intervention for the treatment of life-threatening cardiopulmonary failure, I found time-dependent platelet activation and dysfunction. Plasma matrix metalloproteinase-2 activity was elevated and positively correlated with platelet activation, whereas tissue inhibitors of matrix metalloproteinase-2 in plasma were below detection limits using Western blot analysis.

Inhaled nitric oxide has been proven to be effective to improve systemic oxygenation in critically ill neonates with life-threatening hypoxemic respiratory failure. I demonstrated that inhaled nitric oxide inhibited collagen-induced platelet aggregation via cyclic GMP-dependent mechanisms.

Glutathione, other thiol-containing pharmacological agents and selective inhibitors of matrix metalloproteinase-2 may be novel therapeutic strategies for the management of ischemia-reperfusion injury in the heart or platelet activation and dysfunction in therapies involving an extracorporeal circulation. Understanding the mechanism of platelet dysfunction can help to develop therapies to prevent or reduce hemorrhagic complications during the application of novel interventions such as extracorporeal membrane oxygenation and inhalation of nitric oxide.

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Dedicated to

My wife, Grace

&

My children, Christopher & Douglas

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ABBREVIATIONS

BCNU	1, 3-bis(2-Chloroethyl-)-1-nitrosourea
cyclic GMP	Cyclic 3',5'-guanosine monophosphate
ECMO	Extracorporeal membrane oxygenation
GSH	Glutathione
GSSG	Glutathione disulfide
iNOS	Inducible nitric oxide synthase
MMPs	Matrix metalloproteinases
MPG	*N*-(2-Mercaptoproprionyl)-glycine
L-NMMA	N^G-monomethyl-L-arginine
LVDP	Left ventricular developed pressure
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{\bullet-}$	Superoxide anion
ODQ	1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
RPP	Rate-pressure product
SNAP	S-nitroso-N-acetyl-*d*,*l*-penicillamine

CHAPTER I

INTRODUCTION

1. OXIDATIVE STRESS AND FREE RADICAL INJURY

1.1 Oxidative stress

Birch first described strange reactions precipitated by breathing hyperbaric oxygen which he thought were not explainable on a psychological basis.[1] He described these as "...a sense of constriction of forehead and temples; ... sudden faintness; palpitation of the heart; ... a state of unnatural excitement of the entire nervous and vascular systems, which has continued for successive days". Subsequently, Haldane reported that the immediate effect of suddenly administering high concentrations of oxygen to a hypoxic person was sometimes unpleasant as he had observed from experiments on himself and others; "... the heart may become tumultuous in its action and the breathing irregular...".[2] Haldane advised against too rapid addition of oxygen to the inspired air in cyanosed patients.

The description by Birch and Haldane illustrates the effect of reoxygenation injury to body systems following hypoxia. Meyer provided the first piece of direct evidence that oxygen therapy could alter enzyme activity.[3] He found that suspensions from macerated brain tissue which had been previously exposed to oxygen at pressures slightly less than 4 atmospheres for 4 hr, were less efficient in oxidizing guaiacum in the presence of hydrogen peroxide than those of similar tissue preparations which had been exposed to atmospheric air for an equal period. It was concluded the oxygen therapy could cause a decrease in cerebral metabolic function. Libbrecht and Massart demonstrated the complete inhibition of oxygen consumption in fresh succino-dehydrogenase preparations by oxygen at between 4 and 5 atmospheres.[4] But in experiments in which the activity of the cytochrome system in

the preparation had been previously eliminated by the use of cyanide, this poisoning of the dehydrogenase system was absent. The authors concluded that molecular oxygen was not toxic but it became so when activated by the cytochrome system; according to them it was "active oxygen" which inhibited the dehydrogenase system. Bohr and Bean confirmed similar findings in fresh extracts of porcine hearts.[5]

This reoxygenation injury is related to *oxidative stress*. The chemical definition of oxidation refers to the process resulting in an increase in the positive valence state of an atom or molecule, such as by the removal of one or more electrons. The oxidative process is always coupled with a reduction process which has an equivalent increase in the negative valence state of another atom or molecule. Thus, oxidative or oxidant stress is a disturbance in the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage.[6] It is usually associated with an increased production of reactive oxygen species.[7]

1.2 Production of oxygen-derived radicals

Oxygen-derived radicals refer to a class of chemical compounds with an unpaired electron occupying the outer orbital of the oxygen molecule.[8] Oxygen-derived radicals have variable chemical reactivities depending on their stability in the energetic hierarchy.[9] They are also by-products of cellular metabolism during tetravalent reduction of molecular oxygen to water. Mitochondria are considered as a significant source for the production of oxygen-derived radicals such as superoxide ($O_2^{\bullet -}$, Fig. 1.1, equation 1) under normal physiologic conditions because about 1% of the electron flow in the mitochondrial respiration cascade generates oxygen-derived

radicals.[10] Alternative sources of $O_2^{\bullet-}$ include the cytosolic xanthine oxidase (which converts hypoxanthine to uric acid)[11] and the membrane-bound NAD(P)H-dependent oxidase (the reduction of oxygen to $O_2^{\bullet-}$ and hydrogen peroxide).[12] Superoxide is quickly reduced to hydrogen peroxide by the antioxidant enzyme superoxide dismutase in biological tissues (Fig. 1.1, equation 2). Interestingly, mitochondria can also maintain the cellular redox status by eliminating $O_2^{\bullet-}$ of both mitochondrial and cytosolic origin via both enzymatic and non-enzymatic dismutative mechanisms (superoxide dismutase and protonmotive pump, respectively).[13]

Under physiological conditions, $O_2^{\bullet-}$ is a relatively weak oxidizing agent.[14] Much of the oxidative injury may be related to hydroxyl radical ($OH^{\bullet}$) which, in contrast to $O_2^{\bullet-}$, is an extremely reactive molecule and the most directly cytotoxic species of oxygen-derived radicals.[11] Hydroxyl radical has been shown to be capable of initiating lipid peroxidation in vitro.[15] Indeed, $O_2^{\bullet-}$ has been considered to play an important role in the generation of $OH^{\bullet}$, via the Haber-Weiss reaction (Fig. 1.1, equation 3),[16] and alternatively by the Fenton reaction, which requires the presence of a reduced metal ion such as ferrous or cupric ions (Fig. 1.1, equations 4 and 5). While the Haber-Weiss reaction is too slow to be a biologically important source of $OH^{\bullet}$ in the absence of a catalyst such as iron,[17,18] free iron level is extremely low because it is normally bound to ferritin. Thus it is not certain whether there is sufficient free iron present in biological systems to catalyze the Fenton reaction.[19] Therefore, other oxygen-derived radicals or alternative pathways for the generation of $OH^{\bullet}$ may be responsible for the oxidative damage.

1.3 Free radical injury during ischemia and reperfusion

During myocardial ischemia, the production of oxygen-derived radicals is enhanced. Increased production of $O_2^{\bullet-}$ from the respiratory chain in mitochondria was observed.[20] An increase in intracellular levels of Ca^{2+} as a result of ischemia can also increase the conversion of xanthine dehydrogenase to xanthine oxidase which further increases the production of $O_2^{\bullet-}$.[21] Increased intracellular levels of Ca^{2+} may also activate phospholipase C to stimulate the metabolism of arachidonic acid [22] and catecholamines,[23] which generate oxidant species as by-products. As a result, different kinds of oxidant species are generated, including $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ and $OCl^{\bullet}$ (produced from hypochlorous acid, HOCl, and hydrogen peroxide in the presence of myeloperoxidase and chloride ions).

Oxygen-derived radicals are involved in reperfusion injury following ischemia in different organs including heart,[24] brain,[25] small intestine,[26] liver,[27] kidney,[28] pancreas,[29] skeletal muscle[30] and skin.[31] During reperfusion, oxygen-derived radicals, which peak in concentration within the first min of reperfusion, have been demonstrated in ischemic hearts from different animal species.[32,33,34] The formation of oxygen-derived radicals has been associated with myocardial stunning (an acutely reversible impairment in myocardial contractile function upon recovery from an ischemic or hypoxic insult) following reperfusion of ischemic tissue.[35]

Due to their multiple targets in the cell, the deleterious effects of oxygen-derived radicals are numerous.[15,36,37,38] Deoxyribonucleic acid, protein and lipid are three important targets of oxygen-derived radicals in the cell. Following the exposure to oxygen-derived radicals, mitochondrial, and to a lesser extent nucleosomal,

deoxyribonucleic acids show oxidative damage including base or sugar modification, strand breaks and DNA-protein-crosslinking.[39] Oxidative damage to proteins results in conformational changes, fragmentation, or cross-linking due to chemical modification of amino acid residues and leads to the alteration or loss of enzyme activity and function of ion channels, transporters and receptors. Oxygen-derived radicals cause lipid peroxidation in a process that involves four classical steps: chain initiation, chain propagation, chain branching and termination. The reaction of oxygen-derived radicals with lipids is initiated through their abstraction of a hydrogen atom from the unsaturated fatty acid side chain to form a carbon-centred lipid radical. Double bond rearrangement results in the formation of a more stable conjugated diene which may then react with molecular oxygen to form a peroxy radical. This peroxy radical is highly reactive and is capable of removing hydrogen atoms from adjacent fatty acids, thus propagating the chain reaction of oxidative injury. If lipid peroxidation is allowed to continue unabated, such reactions may result in decreased membrane fluidity, disruption of membrane-bound enzyme and transport systems, alteration of cell permeability and finally cell death.

2. NITRIC OXIDE AND PEROXYNITRITE

2.1 Nitric oxide

Nitric oxide (NO) is generated from a five-electron oxidation of L-arginine catalyzed by NO synthase enzymes (NOS).[40] There are at least three isoforms of NOS which have been named on the basis of the cell types they were originally

discovered in: nNOS from neurons, eNOS from endothelial cells and inducible NOS (iNOS), an isoform expressed in macrophages and many other cell types in response to immunological stimulation.[41,42] Nitric oxide has been shown to be an important physiologic and regulatory mediator that modulates vessel wall tone and hemostatic-thrombotic balance (causing vasodilation and inhibition of platelet adhesion and aggregation, respectively) primarily through the stimulation of the soluble guanylate cyclase.[43,44] Changes in its generation or metabolism contribute to the pathogenesis of vascular and thrombotic disorders. Nitric oxide also accounts for the pharmacological activity of various NO-donating drugs including organic nitrates.[45] Conversely, the inhibition of NOS causes vasoconstriction[40] and potentiates aggregation induced by different aggregating agents in vitro.[46]

2.2 *Signal transduction mechanism of nitric oxide in the heart and platelets*

A substantial part of the NO-activated transduction mechanism depends upon the stimulation of soluble guanylate cyclase which results in the increased synthesis of cyclic GMP.[43,47] Cyclic GMP activates a cyclic GMP-dependent protein kinase and phosphorylates various target proteins,[48] including a vasodilator-stimulated phosphoprotein (VASP, molecular weight 46-50 kDa).[49] Cyclic GMP also decreases basal and stimulated concentrations of intracellular Ca^{2+} levels by modulating the activity of Ca^{2+} handling systems in myocardial cells[50,51] and platelets.[52,53] Moreover, the inhibition of cyclic GMP phosphodiesterase can also result in elevated cyclic GMP levels leading to the enhancement of NO-activated cyclic GMP-dependent effects.[54]

Cyclic GMP-independent effects of NO have also been found in vascular smooth muscle cells and platelets. Nitric oxide may interact with the cyclooxygenase[55] and 12-lipoxygenase enzymes,[56] some enzymes of the mitochondrial respiratory chain,[57] glyceraldehyde-3-phosphate dehydrogenase[58] and thiol-containing proteins such as albumin.[59] However, the significance of cyclic GMP-independent effects of NO on cellular function remains to be established.

2.3 Role of nitric oxide in the physiological function of the heart

Under normal circumstances NO is synthesized by a Ca^{2+}-dependent NOS, now characterized as eNOS, which is localized in endothelial, endocardial and myocardial cells.[60] Nitric oxide regulates coronary perfusion by its vasodilatory action and modulates contractile function by its negative inotropic and chronotropic actions.[61,62] Nitric oxide also causes reversible inhibition of mitochondrial oxygen consumption due to its effect on complex II of the respiratory chain.[57,63] Nitric oxide from endothelial cells can also regulate platelet, leukocyte and cardiac myocyte function in a paracrine manner.[64,65,66]

The physiological role of NO in the heart is protective and multifaceted. NO stimulates soluble guanylate cyclase which reduces intracellular concentration of Ca^{2+} partly through the activation of cyclic GMP-dependent protein kinase.[50,51] The lowered intracellular Ca^{2+} concentration results in vasodilation and decreased contractile function, which could be beneficial in ischemic tissue. Nitric oxide can terminate the chain propagation of lipid peroxidation during oxidative stress.[67] The NO-mediated

inhibition of platelet and leukocyte activation and their subsequent adhesion to the endothelial surface is important for the preservation of coronary perfusion.[64,65]

2.4 *Role of nitric oxide in the physiological regulation of platelet function*

When a blood vessel is damaged and the endothelium is disrupted, platelets are recruited from the circulating blood to form an occlusive plug. Nitric oxide is involved in the modulation of all stages of platelet activation by inhibiting adhesion and aggregation as well as stimulating disaggregation of platelet aggregates both in vitro,[68] and in vivo.[69] Nitric oxide can regulate platelet function in both *autocrine* (NO generated within the platelet) and *paracrine* (NO generated from cells outside of platelets) manners. The autocrine regulation is based on the platelet activation-dependent increase in the activity of eNOS that offsets platelet activation via soluble guanylate cyclase and cyclic GMP-dependent mechanism.[44,47,70]

The paracrine regulation of platelet function is based on the continuous generation, release, and diffusion of NO from endothelial cells to the platelet microenvironment. It is likely that pulsatile flow and changes in shear stress are responsible for the constant generation and release of NO by vascular endothelial cells.[71] Interestingly, pulsatile blood flow and the shear rate are also major determinants of platelet behavior in vivo.[72] Under physiological conditions in whole blood, while the more numerous and larger erythrocytes occupy the axial stream, platelets assume the position close to the endothelial cells. This rheological arrangement allows for the efficient transfer of NO from the endothelium to platelets.

A number of animal and human studies support an important role of NO in the regulation of platelet function in vivo. These studies have demonstrated that: (1) basal or stimulated NO production results in inhibition of platelet aggregation induced by some aggregating agents or endothelial injury[73] and also increases bleeding time,[74] (2) luminal release of NO from human vasculature increases intraplatelet cyclic GMP levels,[75] and (3) the administration of the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) reversibly increases platelet aggregation and granule release in healthy volunteers.[76]

2.5 *Peroxynitrite – the ugly molecule*

Nitric oxide reacts with $O_2^{\bullet-}$ at a diffusion-limited rate ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$) (Fig. 1.1, equation 6) to yield peroxynitrite ($ONOO^-$).[77] The rate constant of formation of $ONOO^-$ is more than three-fold faster than the dismutation of $O_2^{\bullet-}$ by superoxide dismutase ($k = 2 \times 10^9\ M^{-1}\ s^{-1}$) (Fig. 1.1, equation 2). Under physiological conditions, a very small amount of $ONOO^-$ is formed as the concentration of superoxide dismutase is in excess of NO (1 μM vs. 10 nM, respectively). However, in situations with maximal vascular NO production (estimated to be $\geq$ 1 μM),[78] the formation of $ONOO^-$ will predominate over the dismutation of $O_2^{\bullet-}$. Hence, $ONOO^-$ can be formed under pathophysiological conditions, such as ischemia and reperfusion, or when iNOS is expressed in cells during inflammatory disorders. Activated macrophages[79] or neutrophils,[80] neuronal cells,[81] vascular endothelial cells[82] and cardiac myocytes[83] have the capacity to synthesize $ONOO^-$. Peroxynitrite decreases the vascular relaxation response to endothelium-dependent vasodilators[84] and depresses

myocardial contractile function,[85] and stimulates aggregation of human platelets.[86] The endogenous formation of $ONOO^-$ contributes to endothelial injury[82] and myocardial ischemia-reperfusion injury.[87] Furthermore, $ONOO^-$ has also been implicated in the pathogenesis of atherosclerosis,[88] septic shock[89] and neurodegenerative diseases.[90]

Peroxynitrite is a precursor to highly reactive oxidant species. Peroxynitrite has a pKa of 6.8 at 25 °C and is therefore rapidly protonated at physiological pH to form peroxynitrous acid (Fig. 1.1, equation 7).[77] Peroxynitrous acid is highly unstable and its degradation by either heterolytic or homolytic cleavage (Fig. 1.1, equations 8 and 9) results in the formation of strong oxidants, including nitronium ion (NO_2^+) and an intermediate with the character of both $OH^\bullet$ and $NO_2^\bullet$.[91] The effect of $ONOO^-$ is deleterious and extensive by acting on the cell membrane,[92] intracellular proteins[93] and deoxyribonucleic acid[94] leading to structural damage, enzymatic dysfunction or cell death. In particular $ONOO^-$ has been shown to induce lipid peroxidation,[67,95] protein modifications by oxidizing sulfhydryl moieties[96] and nitrating tyrosine residues,[97] and inhibition of mitochondrial respiration.[98] Aconitase is an enzyme in the Krebs cycle responsible for the conversion of citrate to isocitrate and has commonly been used for the study of $ONOO^-$-induced inhibition of enzyme activity.[99,100] It belongs to the family of iron-sulfur-containing dehydratases whose activities depend on the redox state of their cubane [4Fe-4S] cluster[101,102] and cysteine residues around the iron cluster.[103] Nitric oxide was originally reported to inactivate aconitase.[104] However, $ONOO^-$ is markedly more potent and efficacious than NO in inhibiting aconitase.[99,100]

2.6 *Role of nitric oxide in the heart under pathological conditions*

2.6.1 *Nitric oxide-deficient states*

The vasodilator and platelet-regulatory functions of endothelium are impaired with altered levels of formation or biological activity of NO in various vascular diseases, including essential hypertension,[105] diabetes[106] and coronary artery disease.[107] These disorders have characteristic features such as endothelial injury, accumulation of oxidized lipids, platelet and leukocyte activation and can lead to vascular smooth muscle cell proliferation as well as thrombotic and ischemic complications. Thus impaired NO synthesis and action have been implicated in the pathogenesis of these disorders.

2.6.2 *Nitric oxide-excessive states*

Sepsis syndrome (a systemic response to infection with septic shock and multiple organ failure in severe condition) is common in clinical practice. The breakdown products of NO in vivo, serum nitrite and nitrate, are increased in patients with sepsis, especially those with hypotension.[108] It has been shown that iNOS is expressed in a variety of organs during septicemia and septic shock.[109] The invasion of bacteria and exposure of cells to bacterial exotoxin or endotoxin result in the endogenous production of pro-inflammatory cytokines which trigger the expression of iNOS in a wide variety of cells, and thus large amounts of NO are generated. The increased production of NO results in complex effects on the hemostatic-thrombotic balance and microvascular perfusion leading to a progressive loss in vascular reactivity, profound hypotension and endothelial cell damage.[60,110] These detrimental effects of excessive amounts of NO in sepsis can be antagonized by inhibiting NOS with L-

NMMA.[111] Anti-hypotensive effects with reduced vasopressor requirement have been shown in the clinical treatment of septic shock with NOS inhibitors.[111] While no serious, dose-dependent adverse effects of NOS inhibition have been observed, Phase I dose-ranging safety studies have reported dose-limiting side effects consisting of decreased cardiac output and exacerbation of pulmonary artery hypertension.[112] Therefore, the beneficial role of NOS inhibitors in the treatment of septic shock has not been firmly established. Indeed, non-selective NOS inhibitors that inhibit all isoforms of NOS can potentiate cytokine-stimulated platelet adhesion to endothelial cells,[113] precipitate renal glomerular thrombosis[114] and exacerbate sepsis-induced renal hypoperfusion.[115] Thus, generation of an appropriate amount of NO is crucial to maintain the integrity of the microvasculature during sepsis.[116] The effect of $ONOO^-$, which is likely to be generated during sepsis,[89] on the hemostatic-thrombotic balance remains to be elucidated. Moro et al have demonstrated that the effect of $ONOO^-$ on the platelet function depends on its microenvironment; being aggregatory in the absence of thiols and anti-aggregatory (inhibitory) in the presence of thiols.[86] Thus, unraveling the roles of nitrogen- and oxygen-derived radicals species may help to explain some clinical symptoms of sepsis, such as thrombocytopenic-thrombotic phenomenon, myocardial dysfunction and hyporeactivity with poor tissue perfusion.[117]

2.6.3 Role of nitric oxide in ischemia and reperfusion of the heart

The L-arginine-NO pathway plays an important role in ischemia-reperfusion injury[85] and hypoxia-reoxygenation injury.[118] In this pathology, NO is also considered as a double-edged sword exerting both beneficial and detrimental effects. On one hand,

NO is cytoprotective through vasodilator, platelet- and leukocyte- inhibitory effects. On the other hand, NO can be cytodestructive following its conversion to $ONOO^-$. Indeed, $ONOO^-$ formation is increased in the early stage of reperfusion / reoxygenation in the heart,[87] when there is a simultaneous increased production of NO and $O_2^{\bullet-}$.[119] Peroxynitrite causes impaired recovery of myocardial function,[87] endothelial[84] and platelet dysfunction.[86] Indeed, an improvement of myocardial mechanical function during reperfusion has been demonstrated in isolated rat hearts treated with a NOS inhibitor (L-NMMA)[87,120] or a cell-permeable superoxide dismutase mimetic (Mn(III)-tetrakis (4-benzoic acid) porphyrin)[121] to limit the amount of $ONOO^-$ production.

2.7 *Role of nitric oxide in platelet function under pathological conditions*

Nitric oxide is an important player in the pathogenesis of atherosclerosis by inhibiting platelet activation which involves in the atherosclerotic process. Studies have shown that oxidized or low-density lipoproteins decrease the bioactivity of NO by various mechanisms including: (1) inhibition of NOS activity, (2) direct inactivation of NO, (3) changes in NO metabolism, and (4) inhibition of L-arginine uptake which limits the production of NO from iNOS.[122,123,124,125] In contrast, high-density lipoproteins prevent thrombosis by inhibiting platelet function that is associated with increased NOS activity.[126] Interestingly, during atherogenesis there is an increased expression of iNOS in megakaryocytes[127] and this may result in cellular toxicity. Indeed, the mechanism of cytotoxic effects of NO derived from iNOS may be indirect and involve its reaction with $O_2^{\bullet-}$ to form $ONOO^-$[77,128] which stimulates platelet aggregation.[86] The net effect (cytoprotective or cytodestructive) of NO is likely to be dependent on the

microenvironment where both NO and $O_2^{\bullet-}$, and therefore $ONOO^-$, are generated and the ability of antioxidants to combat $ONOO^-$.[88]

3. THE ANTIOXIDANT SYSTEM IN CELLS

3.1 Antioxidants – definition and classification

An antioxidant is defined as a substance, when present in small amounts, inhibits oxidation of the bulk. According to the process of classical lipid peroxidation, there are two broad classes of antioxidants – preventive and chain-breaking. Preventive antioxidants reduce the rate of chain-initiating reactions induced by free radicals. This group includes catalase and glutathione peroxidase. Glutathione (GSH) is a substrate for GSH peroxidase and is classified as a preventive antioxidant. Chain-breaking antioxidants abolish the propagation of free radical chain reactions and include vitamin C (ascorbate) and vitamin E (tocopherols).

3.2 Glutathione – its conventional antioxidant action

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which forms the largest pool of non-protein thiols in the body.[129] Being the major antioxidant in the cell, GSH inactivates free oxygen-derived radicals in the presence of GSH peroxidase.[130] Following the dismutation of $O_2^{\bullet-}$ to hydrogen peroxide, GSH is oxidized to glutathione disulfide (GSSG) by GSH peroxidase (Fig. 1.2, equation 1). GSH is then regenerated from GSSG by GSH reductase. While the intracellular concentration of GSH is important for its antioxidant action, the increased capacity

and turnover rate of the GSH redox cycle has also been postulated to be the primary mechanism for the antioxidant function of GSH.[131] Furthermore, another endogenous thiol, L-cysteine, and thiol-containing agents such as *N*-(2-mercaptoproprionyl)-glycine are important antioxidants which protect cells and tissue from oxidative stress.[132,133]

3.3 Additional antioxidant role of GSH in oxidative stress

Radi et al suggested the antioxidant effect of GSH might be due to the ability of GSH to react with $ONOO^-$ to form GSSG (Fig. 1.2, equation 2).[96] In addition, thiols can also react with $ONOO^-$ to form nitrosothiol derivatives, such as *S*-nitrosoglutathione from the reaction of GSH and $ONOO^-$ (Fig. 1.2, equation 3).[86,134] Vasodilator activity[84] and inhibition of platelet aggregation[86] have been observed when $ONOO^-$ reacts with thiols to form compounds including nitrosothiols which act as donors of NO.[59,84,86,132] This detoxification of $ONOO^-$ may be a unique feature of this class of compounds, as this results in the formation of NO donors that antagonize the detrimental reactions of $ONOO^-$ in biological systems[92-98] or have cytoprotective actions on the cell through NO-mediated stimulation of soluble guanylate cyclase and other mechanisms.[135]

4. SUPPLEMENTATION OF GLUTATHIONE IN ISCHEMIA-REPERFUSION OF THE HEART

While hearts depleted of GSH had a greater susceptibility to ischemia-

reperfusion injury,[136] the effect of exogenous GSH on recovery of myocardial function following ischemia-reperfusion or hypoxia-reoxygenation remains controversial.[137,138] Tani did not find any protective action of thiols such as GSH, cysteine, and N-acetylcysteine in myocardial ischemia-reperfusion injury in isolated rat hearts.[137] In contrast, Seiler et al demonstrated cardioprotective effects of GSH or GSH mono-ethyl ester in isolated rat hearts subjected to intermittent periods of hypoxia.[138] The lack of protective action with in Tani's experiment could be related to the millimolar concentrations of GSH used because GSH has cardiodepressive effect at this range of concentrations.[137] A concentration-response study of GSH on the recovery of cardiac function is required and should include micromolar concentrations of GSH. The mechanism by which GSH may mediate its protective effects in the heart needs further investigation.

During the first min of reperfusion of the ischemic heart there is a burst of $ONOO^-$ production that contributes to the impairment of myocardial function.[87] The production of $ONOO^-$ and its detrimental effects in the heart are blocked by low concentrations of inhibitors of NOS.[87,120] In addition, infusion of a nitrosothiol which is a NO donor, *S*-nitroso-N-acetyl-d,l-penicillamine, also protected the heart, likely due to the ability of NO to interfere with the oxidative reactions of endogenously formed $ONOO^-$.[67,86,87,139] Thiols can protect against $ONOO^-$-induced inhibition of metabolic enzyme activity such as aconitase and this may be in part related to its protective role against myocardial stunning.[103,104] It is possible that GSH may act as a unique scavenger of $ONOO^-$ through the formation of a NO donor intermediate, and thus improve the recovery of mechanical function in ischemia-reperfusion injury.

5. MATRIX METALLOPROTEINASES IN THE HEART AND PLATELETS

5.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) belong to a family of zinc neutral endopeptidases.[140] The MMPs are synthesized in a latent form (pro-enzyme, pro MMP) and require activation by proteolytic cleavage of an amino-terminal domain,[141] or conformational changes induced by oxidative stress.[142] They are released from intracellular sites into extracellular space. Matrix metalloproteinases are involved in the remodeling of the extracellular matrix of tissue during various physiological and pathological conditions such as embryonic development, inflammation and cancer.[143] The expression of MMPs has been shown to be upregulated during the pathological remodeling processes. Following myocardial infarction increased activities of collagenase and other neutral proteinases have been shown,[144] which may be responsible for the rapid degradation of extracellular matrix collagen in myocardial infarction.[145] Recently, enhanced expression and increased activity of myocardial MMPs have been shown to contribute to ventricular remodeling in pigs with pacing-induced heart failure.[146]

5.2 Matrix metalloproteinase-2 – its dual physiological roles

Amongst the metalloproteinases, MMP-2 (pro-enzyme: pro MMP-2, progelatinase A, 72-kDa; active enzyme: MMP-2, gelatinase A, 64 kDa) is involved in the degradation of collagen and intact collagen type IV, a major component of the basement membrane. Matrix metalloproteinase-2 has been shown to be involved in

ventricular remodeling such as congestive heart failure.[146] Recently, MMP-2 was found in the releasate of human platelets during collagen-stimulated aggregation.[147] Sawicki and his colleagues have shown that this platelet-derived enzyme mediates a novel pathway of platelet aggregation.[147] Hence, MMP-2 plays dual physiological roles: chronically in the remodeling of connective tissue and acutely in the regulation of platelet aggregatory function. Tissue inhibitors of MMPs (TIMPs) are endogenous proteins which provide specific inhibition of MMPs.[148] The biological status of MMPs activity relies on the balance between MMP-2 and TIMP-2, the latter binds to MMP-2 and inhibits collagen-stimulated platelet aggregation.[147,149]

While MMPs have been found in a variety of ventricular remodeling conditions, the expression of MMP-2 and the acute release of MMP-2 from the heart under normal conditions are not known. Substances such as NO and prostaglandin have dual biological actions on platelets as well as the heart. Therefore, based on the acute role of MMP-2 in the regulation of platelet aggregation, it is possible that MMP-2 may also play an acute role in ischemia-reperfusion of the heart. No information is available regarding the expression and the release of MMP-2 from the heart following ischemia and reperfusion. It is interesting to note that $ONOO^-$ can activate pro MMP-2 from stimulated neutrophils[142] as well as inactivate TIMP-2 activity in vitro.[150] It is possible that $ONOO^-$ can induce conformational changes with the modification of cysteine residues and sulfhydryl groups at the active sites of pro MMP-2 and TIMP-2.[142] The rapid production of $ONOO^-$ during early reperfusion in ischemic hearts may activate pro MMP-2 which could possibly be involved in the re-establishment of normal coronary flow and mechanical contractile function.

6. EXTRACORPOREAL MEMBRANE OXYGENATION – A MODEL OF PLATELET ACTIVATION AND DYSFUNCTION

Extracorporeal membrane oxygenation (ECMO) has been widely used to provide cardiopulmonary support for neonates with severe respiratory failure.[151] Following cannulation of jugular vessels, hypoxemia is corrected by the reinfusion of well-oxygenated blood (oxygen partial pressure > 300 mmHg) back to the systemic circulation. During ECMO, blood is exposed both to hyperoxia and large artificial surfaces (silicone membrane oxygenator and polyvinyl tubing) which can impose a substantial activating stimulus to the platelet.[152] Indeed, ECMO is associated with increased levels of various cytokines and endotoxin (secondary to bacterial translocation from the intestine).[153] Platelet activation during cardiopulmonary bypass can contribute to hemorrhagic and thromboembolic complications.[154] While thrombocytopenia with abnormal platelet aggregatory function during ECMO has been reported,[155] the direct evidence and temporal changes of platelet activation are lacking. Moreover, there is no information on the role and changes of plasma MMP-2, which is released extracellularly and involved in the regulation of platelet aggregation,[147] in critically ill neonates. Understanding the mechanism of platelet activation and dysfunction in the extracorporeal circulation is important to design pharmacological agents to alleviate or prevent platelet dysfunction in patients who undergo cardiopulmonary bypass, hemodialysis, as well as ECMO. The use of MMP-2 inhibitors will open a novel therapeutic avenue in the management of these patients at risk for the development of hemorrhagic complications.

7. INHALED NITRIC OXIDE AND PLATELET FUNCTION IN NEONATES

7.1 Clinical use of inhaled nitric oxide in neonates

In the vasculature, generation of NO regulates vascular wall reactivity and platelet function primarily through the stimulation of soluble guanylate cyclase.[156] As some vascular disorders are associated with decreased production of NO or enhanced metabolism of NO, the pharmacological supplementation of endogenous NO with NO donors or inhaled NO gas is an attractive therapeutic strategy in these disorders.[107]

Inhalation of NO gas has been used as one of the therapeutic approaches to the treatment of lung disorders associated with hypoxia and pulmonary vasoconstriction.[157] The concept of selective pulmonary vasodilation following inhaled NO[158] is based on its direct access to the pulmonary vasculature and a very short half-life of NO gas in biological milieu[67] that apparently precludes its effects on the systemic circulation. Inhaled NO has been used increasingly in neonates following reports on improvement in oxygenation in term neonates with severe hypoxemia.[159,160] This treatment can increase systemic oxygenation by improving ventilation-perfusion matching.[161] Inhaled NO has also been used in premature infants with suspected hypoplastic lungs and intractable hypoxemia unresponsive to aggressive ventilatory support and surfactant treatment.[162] The studies by Finer et al have showed that the beneficial effects of inhaled NO (2 - 20 ppm) in critically ill neonates may depend, in part, on improved ventilation-perfusion matching.[161,163] Recently, two double-blinded, randomized controlled trials have documented the efficacy of inhaled NO in improving systemic oxygenation and reducing the requirement for ECMO in critically

ill neonates with severe hypoxemia, with or without pulmonary hypertension.[164,165] Both studies indicated a low incidence of adverse effects such as methemoglobinemia or increase in nitrogen dioxide concentrations.

7.2 Platelet function during inhaled nitric oxide therapy

One of the main advantages of the use of inhaled NO is its short biological half-life. It has been postulated that inhaled NO should act only locally in the pulmonary circulation at the level of vessel wall and will not exert significant systemic effects due to its rapid inactivation by red cell oxyhemoglobin.[67] However, platelets, in contrast to erythrocytes, are in close contact with the pulmonary vascular endothelium and therefore platelet function could also be affected by inhaled NO. Nitric oxide inhibits platelet adhesion and aggregation in vitro with the stimulation of guanylate cyclase.[166] However, its antiplatelet activity is limited by short half-life (sec) in biological milieu.[67] Hogman et al reported a prolongation of the bleeding time in normal adults who received inhaled NO,[167] and also demonstrated a prolonged bleeding time in rabbits receiving as little as 3 ppm of inhaled NO.[168] Recently, Samama et al have shown a decrease in collagen-induced platelet aggregation in a study of 6 critically ill adults with acute respiratory distress syndrome treated with inhaled NO.[169] In critically ill neonates, the metabolism of NO can be affected by poor oxygenation, alterations of antioxidant stores[170] and increased sensitivity of fetal platelets to inhibition by NO.[171] Hence, the biological half-life of NO can be prolonged in critically ill neonates treated with inhaled NO.

7.3 Clinical consequences of inhaled nitric oxide in neonates

Bleeding is an important complication that affects mortality and long-term neurodevelopmental outcome in critically ill neonates. Intracranial hemorrhage is a common finding in premature infants, and it has been reported to occur in approximately 40 % of infants with birth weight less than 1500 g.[172] Platelet dysfunction contributes to the precipitation or aggravation of intracranial hemorrhage.[173] Hemorrhage and deranged platelet aggregation have been shown in neonates with septicemia.[174,175] In a review of the Extracorporeal Life Support Organization Registry, Horwitz et al found that culture-positive, septic neonates who require extracorporeal membrane oxygenation appear to be at an increased risk for intracranial hemorrhage and death.[176] Intracranial hemorrhage appears to be the primary factor affecting survival in these patients. In these high-risk infants whose platelet function is already compromised by the existing hypoxemia and acidemia,[177,178] the risk of bleeding during inhaled NO therapy may be exaggerated and lead to the development of intracranial hemorrhage. Inhaled NO therapy could therefore be potentially hazardous for its use in these neonates at risk for intracranial hemorrhage. On the other hand, thrombotic complications such as oxygenator thrombi and hemofilter malfunction occur more often in those neonates who develop sepsis during ECMO.[179] Therefore, inhaled NO may also have a beneficial role by inhibiting platelet activation in this condition.[180]

8. OBJECTIVES AND HYPOTHESES

In this thesis, I evaluated the beneficial and adverse effects of several novel interventions in the management of ischemia-reperfusion injury in an experimental model of myocardial stunning and as well as the treatment of clinical hypoxemia in critically ill neonates.

1. Glutathione: a novel coronary vasodilator and protective agent in models of $ONOO^-$-mediated injury

A variety of studies have demonstrated the basal production of both NO and $O_2^{\bullet-}$, which react at a diffusion-limited rate to form $ONOO^-$, in the heart. Peroxynitrite can react with thiols to form compounds including nitrosothiols with vasodilator activity by subsequently releasing NO. I studied the action of GSH on coronary flow and mechanical function of isolated rat hearts under aerobic perfusion and following ischemia and reperfusion. Since aconitase is an important enzyme for mitochondrial respiration and is susceptible to inhibition by $ONOO^-$, the effect of GSH on $ONOO^-$-induced inhibition of aconitase activity was also studied. The following hypotheses were tested:

1.1 GSH will relax the coronary vasculature via a NO-dependent mechanism.

1.2 GSH and other thiols will protect myocardial aconitase activity from inhibition by $ONOO^-$ in a concentration-dependent fashion and their protective action will depend upon a free sulfhydryl group.

1.3 GSH will improve the recovery of mechanical function during reperfusion following ischemic injury in isolated rat hearts via scavenging $ONOO^-$ with

the formation of a NO donor intermediate, measured by the stimulation of soluble guanylate cyclase.

2. *Matrix metalloproteinase-2: a novel mediator of myocardial ischemia-reperfusion and platelet aggregation*

While MMP-2 has been shown to be involved in the process of tissue remodeling in various pathophysiological conditions, little is known about the acute role of MMP-2 in the regulation of heart function during ischemia-reperfusion and platelet function during platelet activation. Whether there is an acute release of MMP-2 from the heart under normal and ischemia-reperfusion conditions is unknown. Using an isolated rat heart model I investigated the release of MMP-2 into the coronary circulation and its role in the recovery of mechanical function during ischemia-reperfusion. I also studied the time-course and mechanism of platelet activation during ECMO and its relationship with plasma MMP-2 levels in critically ill neonates. The following hypotheses were tested:

2.1 *MMP-2 is released from the heart into the coronary circulation during aerobic perfusion, and its release will be increased and accompanied by enzyme-activation following ischemia-reperfusion. Inhibition of MMP-2 activity will improve the recovery of mechanical function following ischemia and reperfusion.*

2.2 *Following the initiation of ECMO, platelets will be activated and will release MMP-2 which will contribute to platelet dysfunction.*

3. *Inhaled NO: a novel therapeutic intervention that inhibits platelet function*

Inhaled NO has been increasingly used in the management of neonates with hypoxic respiratory failure with minimum systemic side-effects being reported. However, inhaled NO may inhibit platelet aggregation and thus, increase the risk of intracranial hemorrhage in critically ill neonates. I therefore tested the hypothesis that *inhaled NO will inhibit platelet aggregation in critically ill neonates.*

Fig. 1.1

Reactions leading to generation of oxygen-derived radicals

1. Reduction of molecular oxygen to superoxide anion ($O_2^{\bullet-}$)

$$O_2 + e^- \Rightarrow O_2^{\bullet-} \qquad (1)$$

Possible enzymatic sources of $O_2^{\bullet-}$:

1. NADH dehydrogenase (mitochondria)
2. NAD(P)H oxidase (plasma membrane)
3. Xanthine oxidase (cytosolic)

2. Dismutation of superoxide anion by superoxide dismutase

$$O_2^{\bullet-} + 2H^+ \Rightarrow H_2O_2 + O_2 \qquad (2)\ (k = 2 \times 10^9\ M^{-1}\ sec^{-1})$$

3. Haber-Weiss reaction

$$O_2^{\bullet-} + H_2O_2 \Rightarrow O_2 + HO^- + OH^{\bullet} \qquad (3)$$

4. Fenton reaction

$$O_2^{\bullet-} + M^{n+1} \Rightarrow O_2 + M^n \qquad (4)$$

$$M^n + H_2O_2 \Rightarrow M^{n+1} + HO^- + OH^{\bullet} \qquad (5)$$

M : Cu or Fe

Fig. 1.1 (continued)

Reactions leading to generation of oxygen-derived radicals

5. Formation and fate of peroxynitrite ($ONOO^-$)

Formation : $O_2^{\bullet-} + NO \Rightarrow ONOO^-$ (6) ($k = 6.7 \times 10^9\ M^{-1}\ sec^{-1}$)

Fate: at physiological pH

$ONOO^- + H^+ \Rightarrow ONO\text{-}OH$ (peroxynitrous acid) (7)

Heterolytic → $NO_2^+ + OH^-$ (8)

Homolytic → Cu/Fe → $NO_2^{\bullet} \bullet\bullet\bullet OH^{\bullet}$ (9)

Fig. 1.2

Antioxidant actions of glutathione (GSH)

1. Reaction with hydrogen peroxide (H_2O_2) catalyzed by GSH peroxidase

$$2GSH + H_2O_2 \Rightarrow GSSG + 2H_2O \qquad (1)$$

GSSG : glutathione disulfide

2. Reactions with peroxynitrite ($ONOO^-$)

$$2GSH + ONOO^- \Rightarrow GSSG + 2H^+ \qquad (2)$$

$$GSH + ONOO^- \Rightarrow GSNO + H^+ \qquad (3)$$

Oxidation and nitrosation of GSH depend on relative concentrations of NO and $O_2^{\bullet-}$, the presence of CO_2, pH and temperature.

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CHAPTER II

GLUTATHIONE CAUSES CORONARY VASODILATION IN RAT HEARTS VIA A NITRIC OXIDE AND SOLUBLE GUANYLATE CYCLASE DEPENDENT MECHANISM

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ABSTRACT

The actions of thiols on coronary vascular tone in the intact heart are unknown. Glutathione (GSH), glutathione disulfide (GSSG), L-cysteine (L-cys) (10-1000 μM each), and GSH ethyl ester (3-300 μM) were infused into isolated rat hearts perfused with Krebs-Henseleit solution at constant pressure by the Langendorff method. Glutathione, GSSG and GSH ethyl ester, but not L-cys, caused a concentration-dependent increase in coronary flow with the following order of effectiveness: GSH ethyl ester > GSH = GSSG. The nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 300 μM), prevented the increase in coronary flow to GSH and attenuated that to GSSG (300 μM each). The vasodilation with GSH or GSSG and the associated increase in myocardial cyclic GMP were abolished by 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ, a specific inhibitor of soluble guanylate cyclase) at 1 or 3 μM, respectively. The vasodilator action of GSH was abolished by superoxide dismutase (50 U/ml). Inhibition of GSH reductase abolished GSSG-induced vasodilation. Neither glybenclamide (1 μM) nor indomethacin (4 μM) affected the vasodilator action of GSH and GSSG. I conclude that GSH and GSSG cause coronary vasodilation which is mediated by a nitric oxide and guanylate cyclase-dependent mechanism, possibly mediated by the reaction between GSH and peroxynitrite to form S-nitrosoglutathione, a nitric oxide donor.

INTRODUCTION

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which forms the largest pool of non-protein thiols in the body.[1] Being the major antioxidant in the cell, GSH inactivates free oxygen radicals in the presence of GSH peroxidase.[2,3] While the oxidation of GSH to glutathione disulfide (GSSG) by GSH peroxidase is important for its antioxidant action, the increased capacity and turnover rate of the GSH redox cycle has been postulated to be the primary mechanism for the antioxidant function of GSH.[4]

Nitric oxide (NO) plays an important role as an endogenous vasodilator in the control of coronary vascular tone through the stimulation of the soluble guanylate cyclase.[5,6] A vasorelaxant response to GSH and GSSG has been reported in isolated coronary arteries.[7] However, their vascular action has not been studied in the intact coronary circulation and the mechanism of action is poorly understood. As peroxynitrite ($ONOO^-$), the reaction product of NO and superoxide ($O_2^{\bullet-}$), reacts with thiols to form compounds including nitrosothiols with vasodilator activity[8,9,10,11] I, therefore, tested the actions of GSH and related compounds on the coronary flow in aerobically perfused, isolated rat hearts. The data suggest that GSH relaxes the coronary vasculature via a NO and soluble guanylate cyclase-dependent mechanism.

MATERIALS AND METHODS

This investigation conforms with the Guide to the care and use of experimental animals published by the Canadian Council on Animal Care (revised 1993).

Materials

N^G-monomethyl-L-arginine hydrochloride (L-NMMA) and D-NMMA hydrochloride were gifts from the Wellcome Research Laboratories (Beckenham, Kent, UK). 1*H*-[1,2,4] oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, an inhibitor of guanylate cyclase[12]) was purchased from Tocris Cookson Inc. (St. Louis, MO, USA). All other reagents used for perfusion were obtained from Fisher Scientific Ltd. or Sigma Chemical Company. L-arginine or D-arginine were dissolved in 0.1 M Hepes and corrected to pH 7.4 with 10 M HCl. Glybenclamide and ODQ were dissolved in DMSO and diluted with water so that the final concentration of DMSO infusion reaching the heart never exceeded 0.1 % (v:v). A solution of indomethacin was prepared by adding saline to a lyophilized, water soluble preparation of indomethacin (Indocid, Merck Sharp & Dohme, Quebec, Canada). 1, 3-bis(2-Chloroethyl-)-1-nitrosourea (BCNU) was purchased from Bristol-Myers Squibb Canada Inc. (Montreal , Quebec, Canada) and it was dissolved in 10 mM trisodium citrate buffer (pH 4). All other compounds were either dissolved in deionized water or saline.

Heart Preparation and Perfusions

Male Sprague-Dawley rats (250-300 g) were anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg). Hearts were rapidly excised and briefly rinsed by immersion into an ice-cold Krebs-Henseleit solution. They were perfused via the aorta at a constant pressure of 60 mmHg with Krebs-Henseleit solution at 37 °C. The composition of Krebs-Henseleit solution was (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), CaCl (3.0), $NaHCO_3$ (25), glucose (11), and EDTA (0.5), and it was continuously gassed with 95 % O_2 / 5 % CO_2 (pH 7.4). The heart was surrounded by a water-jacketed glass chamber which maintained its temperature at 37 °C.

Spontaneously beating hearts were used in these experiments. A small latex balloon (size 4, Radnoti Glass Technology, Monrovia, CA, USA) connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve. The balloon was filled with deionized water (0.3-0.5 ml) to achieve a left ventricular end diastolic pressure of 8-12 mmHg. The transducer was connected to a Grass VI polygraph on which heart rate and left ventricular pressure were monitored. Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and end diastolic pressures of the left ventricle. The rate-pressure product (RPP) was calculated as the product of heart rate and LVDP and used as a measurement of cardiac mechanical function. Coronary flow was measured by an in-line ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) positioned proximal to the perfusion cannula.

The intervals between thoracotomy and attachment of the heart to the perfusion system and between thoracotomy and beginning of stabilization period were less than 1 and 5 min, respectively. Using this perfusion protocol, hearts maintained a steady state of coronary flow, heart rate and LVDP for at least 80 min after stabilization (time control group, n = 6, see Table 1).

Experimental protocols

Following 20 min of stabilization, drug solutions (see below) were infused into aerobically perfused hearts via a side-port 15 cm proximal to the aortic cannula. They were infused at a constant rate of 0.1 ml/min by a syringe pump (Sage Instruments, Cambridge, MA, USA) and the concentration of the stock solutions were prepared based on an average coronary flow of 14 ml/min. The pH of Krebs-Henseleit solution, measured in control experiments by the use of colorpHast indicator strips (pH 5-10) (EM Science, Gibbstown, NJ, USA) placed in the aortic cannula line and in the coronary effluent, was not altered by any of the drug infusions.

Concentration-dependent vasodilator response of GSH and related compounds

Six to eight hearts were used in each group for the study of concentration-dependent responses of GSH, GSSG and L-cysteine (10-1000 μM for each) and GSH ethyl ester (3-300 μM). The infusion at each concentration lasted for 10 min before the next higher concentration was tested. Coronary flow, heart rate and LVDP were recorded and RPP was calculated at 1, 2, 5 and 10 min during the infusion at each concentration, by which time changes in functional parameters had either plateaued or

reached near steady-state conditions. The recovery of hemodynamic parameters was recorded at 5, 10 and 15 min after stopping the infusion of the highest concentration.

Mechanism of vascular action of GSH and GSSG

The effect of the following drugs or their vehicles (see Materials) was studied during a continuous 30-min infusion, whereby GSH or GSSG were co-infused continuously at 300 μM for the final 15 min:

a) NO synthase inhibitor L-NMMA (50 or 300 μM)

b) D-NMMA (300 μM)

c) ATP- sensitive potassium channel antagonist glybenclamide (1 μM)

d) cyclooxygenase inhibitor indomethacin (4 μM)

e) soluble guanylate cyclase inhibitor ODQ (1 or 3 μM)

f) copper-zinc superoxide dismutase (50 U/ml)

Coronary flow, heart rate and LVDP were recorded before and 15 min after the infusion of the test agent, and 15 min after the co-infusion of the test agent and GSH or GSSG.

To study the reversibility of the effects of L-NMMA, a separate series of hearts were subjected to the infusion of 300 μM L-NMMA for a total of 30 min. For the first 15 min, L-NMMA alone was infused, whereas for the 15-30 min period, GSH or GSSG (300 μM) and L- or D- arginine (1000 μM) were co-infused with L-NMMA.

A further set of experiments was performed to study the dependency upon GSH reductase for the vascular actions of GSH and GSSG. After stabilization, the GSH reductase inhibitor BCNU (150 μM) or its vehicle (10 mM trisodium citrate buffer, pH 4) were infused for 30 min. Five min after discontinuing BCNU infusion, either 300 μM GSH or GSSG were infused for 10 min. Coronary flow, heart rate and LVDP were recorded immediately prior to, and at the end of, thiol infusion.

Assay of myocardial cyclic 3',5'-guanosine monophosphate (cyclic GMP)

Hearts were perfused for 15 min with or without 1 or 3 μM of ODQ, followed by the co-infusion of GSH or GSSG (300 μM each). The hearts were freeze-clamped with tongs cooled to the temperature of liquid nitrogen after 2 min infusion of either GSH or GSSG and stored at -80 °C. Hearts perfused for an equivalent time duration were used as controls. Three hearts were also infused for 2 min with 0.1 μM of acetylcholine and were freeze-clamped.

Freeze-clamped hearts were pulverized under liquid nitrogen with a mortar and pestle. The powdered tissue (200-250 mg) was suspended in 0.5 ml of a solution of ice-cold 100 mM Hepes: 5 mM EDTA: 2.2 % perchloric acid and kept in ice for 15 min. The heart extracts were centrifuged (10,000 g, 2 min at 4 °C) and the supernatant was separated. 250 μl of the supernatant was neutralized with 40 μl of a 1.1 M K_3PO_4 solution. This was centrifuged again (10,000 g, 2 min at 4 °C) and the supernatant was used for assay of cyclic GMP with an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The pellet from the

Hepes:EDTA:perchloric acid extract was kept for protein analysis. This was dissolved by boiling in 0.5 ml NaOH (2 M) for 10 min and then neutralized with 0.5 ml HCl (2 M). This was centrifuged (10,000 g, 2 min at 4 °C) and the supernatant was used for the determination of protein content by bicinchoninic acid reagent, using bovine serum albumin as a standard. The cyclic GMP levels in the myocardial tissue were calculated as pmol/mg of protein.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of the mean. For statistical analysis, one-way ANOVA or one-way repeated measures ANOVA, followed by Fisher's least significant difference test, were used to compare individual means. In experiments with BCNU, a paired *t*-test was used to compare the coronary flow values immediately prior to and at the end of thiol infusion in BCNU or vehicle treated hearts. $P < 0.05$ was considered statistically significant.

RESULTS

1. Effects on coronary flow and mechanical function

Infusion of GSH (10-1000 μM) caused a concentration-dependent increase in coronary flow with a threshold concentration of 30 μM and the maximum change at 1000 μM (from baseline of 13.7 ± 0.6 to 17.7 ± 0.8 ml/min; 29% increase, n = 7, Fig. 2.1A). The maximum change in coronary flow was seen within 5-10 min following a change in GSH concentration. The increase in coronary flow by GSH was reversible, as coronary flow returned to baseline levels within 10 min of the cessation of 1000 μM GSH infusion (Fig. 2.1A). Pilot experiments showed that although 3000 μM GSH (n = 5) or GSSG (n = 3) caused a further increase in coronary flow, this was accompanied by a substantial and irreversible decrease in heart rate, LVDP and RPP (data not shown).

Compared with a time control group, GSH (10-1000 μM) caused no significant effect on heart rate or RPP, whereas only at the highest concentration tested (1000 μM), there was a significant decline in LVDP which was reversible after stopping the infusion (Table 1).

GSSG (10-1000 μM) caused a concentration-dependent increase in coronary flow which was similar to that of GSH (Fig. 2.1B). The threshold concentration was approximately 30 μM and a maximum increase in coronary flow occurred at 1000 μM (from baseline of 13.8 ± 0.6 to 16.8 ± 0.7 ml/min; 22 % increase, n = 6). This increase in coronary flow was not significantly different from that of GSH at 1000 μM. GSSG also caused no changes in heart rate or RPP but caused a significant

decrease in LVDP only at 1000 μM which was reversible after stopping the infusion (Table 1).

The cumulative concentration-response curves to GSH, GSSG and related compounds on changes in coronary flow are shown in Fig. 2.2. Glutathione ethyl ester (3-300 μM) was more effective than GSH or GSSG as a coronary vasodilator. The threshold concentration for GSH ethyl ester was 3 μM and the maximum increase in coronary flow at 300 μM was equivalent to that of 1000 μM GSH or GSSG. In marked contrast to the effect of GSH, GSSG or GSH ethyl ester, no significant changes in coronary flow were observed during infusion of L-cysteine (10-1000 μM, Fig. 2.2), nor were there any changes in heart rate, LVDP or RPP (data not shown).

2. Dependency on NO for the vascular action of GSH and GSSG

During 15-min infusion of L-NMMA (50 or 300 μM), a significant decrease in coronary flow was observed (Fig. 2.3). The vasodilation induced by 300 μM GSH was attenuated by 50 μM and abolished by 300 μM L-NMMA (Fig. 2.3A). The vasodilation with 300 μM GSH following 15 min of 300 μM D-NMMA infusion was not different from that of 300 μM GSH alone (n = 3, data not shown). The vasodilation with 300 μM GSSG was attenuated but not abolished by 300 μM L-NMMA (Fig. 2.3B). The inhibitory action of 300 μM L-NMMA on GSH- or GSSG-induced vasodilation was reversed by 1 mM L-arginine but not by 1 mM of D-arginine (Fig. 2.4).

Glybenclamide (1 μM, n = 4) or indomethacin (4 μM, n = 3) had no significant effect on coronary flow. The increase in coronary flow by 300 μM GSH was not affected by either of these agents (Table 2).

3. Activation of soluble guanylate cyclase for the vascular action of GSH or GSSG

ODQ (1 μM) caused a modest but insignificant reduction in the baseline coronary flow in either the GSH- (n = 8) or GSSG- (n = 4) treated groups (Fig. 2.5). However, if the coronary flow data were combined from these two groups, there was a significant 8% reduction in baseline coronary flow from 13.7 ± 0.2 to 12.6 ± 0.5 ml/min ($P < 0.05$, n = 12). In hearts treated with 3 μM ODQ, coronary flow was significantly reduced (12.5 %) from 13.6 ± 0.2 to 11.9 ± 0.6 ml/min ($P < 0.05$, n = 5). ODQ (1 μM) abolished the vasodilation with 300 μM GSH (Fig. 2.5A). While 1 μM of ODQ partially inhibited the vasodilation with 300 μM GSSG, 3 μM ODQ was required to abolish it (Fig. 2.5B).

Two min infusion of either GSH or GSSG (300 μM each) caused almost a doubling of myocardial cyclic GMP levels. At this time of infusion, coronary flow was significantly increased by 11.5 % and 10.8 % from baseline levels, respectively ($P < 0.05$, n = 3 in each group). The increase in cyclic GMP by GSH or GSSG was abolished by ODQ, 1 μM or 3 μM, respectively (Fig. 2.6). In comparison to the effect of GSH and GSSG, there was a 2.8-fold increase in cyclic GMP levels in hearts infused for 2 min with 0.1 μM acetylcholine (5.0 ± 1.2 pmol/mg protein versus 1.8 ± 0.04 pmol/mg protein in controls, $P < 0.05$, n = 3 in each group).

4. Dependency of $O_2^{\cdot-}$ for the vascular action of GSH

Infusion of copper-zinc superoxide dismutase (50 U/ml) did not significantly alter baseline coronary flow, however, it abolished the vasodilation with 300 μM GSH ($P < 0.05$, $n = 3$) (Fig. 2.7).

5. Dependency of reduced state of GSH for the vascular action of GSH or GSSG

The inhibitor of GSH reductase, BCNU (150 μM), had no significant effect on coronary flow itself, but abolished the increase in coronary flow caused by 300 μM GSSG ($P < 0.05$, $n = 3$), but not by 300 μM GSH ($n = 3$) (Fig. 2.8).

DISCUSSION

This is the first report demonstrating the vasodilator action of GSH and GSSG on the coronary vasculature in the intact heart. The vasodilator action of GSH or GSSG is via a NO- and soluble guanylate cyclase-dependent mechanism as indicated by several lines of evidence: a) their vasodilator effect was inhibited by the NO synthase inhibitor, L-NMMA, b) myocardial cyclic GMP levels were increased in association with their vasodilator effect and, c) the vasodilator action and the associated increase in cyclic GMP levels were prevented by ODQ, a specific inhibitor of soluble guanylate cyclase.

The findings with L-NMMA demonstrated the dependency on NO for the vasodilator actions of GSH and GSSG. L-NMMA abolished the vasodilation with GSH and GSSG in a concentration-dependent and stereoselective manner. The inhibitory effect of L-NMMA was reversed by an excess of L-arginine, but not D-arginine, confirming the selectivity of its action through the inhibition of NO biosynthesis.[13] Moreover, GSH- or GSSG- induced vasodilation was not due to the activation of ATP-dependent potassium channels or the formation of prostaglandins as glybenclamide and indomethacin, respectively, had no effect. The vasodilator action of GSH or GSSG was associated with a doubling of myocardial cyclic GMP levels, and both were abolished by a specific inhibitor of soluble guanylate cyclase, ODQ.[12] These results support a NO-and guanylate cyclase-dependent mechanism for GSH- and GSSG-induced vasodilation.

The vasodilator effect of GSSG showed some significant differences to that of GSH. Firstly, L-NMMA had a weaker inhibitory effect on the vascular action of

GSSG than GSH. Moreover, a higher concentration of ODQ was necessary to completely block both the vasodilator effect of GSSG and the associated increase in cyclic GMP levels. GSSG is taken up into cells by a carrier-mediated mechanism[14] and converted to two molecules of GSH by a NADPH-dependent reduction catalyzed by GSH reductase. Indeed experiments with the GSH reductase inhibitor BCNU, which abolished GSSG-, but not GSH- induced vasodilation, strongly support that reduction of GSSG is a prerequisite to its vascular action. Hence, a difference in the effective concentration of GSH may explain the differential inhibition of L-NMMA and ODQ on vasodilation induced by GSH and GSSG. There was a component of GSSG-induced vasodilation which was not blocked by 300 μM L-NMMA (Fig. 2.3B). However, 300 μM L-NMMA does not completely inhibit NO-dependent relaxations in intact blood vessels[13] and concentrations higher than 300 μM cause a significant change in pH in Krebs-Henseleit solution.[15] As GSSG-induced vasodilation and increase in cyclic GMP were both abolished by ODQ, this suggests that a complete inhibition of NO synthase activity may have been necessary, not that some component of GSSG's action was NO-independent.

It has been widely suggested that the plasma membrane is impermeable to extracellular GSH.[1] However, studies with vascular endothelial cells have shown significant increases in the intracellular concentration of GSH in the presence of extracellular GSH.[16,17] Moreover, a carrier-mediated mechanism has been reported to transport GSH across the blood-brain barrier[18] in addition to the transport mechanism mediated by gamma-glutamyl transpeptidase.[3] An exogenous supply of GSH has significant biological effects in endothelial cells as Suttorp et al

demonstrated that GSH at extracellular concentrations which did not increase intracellular GSH levels reduced hyperoxia-induced injury.[16] Glutathione ethyl ester is known to be more permeable to cells than GSH or GSSG and, following the action of esterases in the cell, increases the intracellular concentration of GSH.[19,20] The higher effectiveness of GSH ethyl ester than either GSH or GSSG also support an intracellular location for the vasodilator mechanism of GSH or GSSG.

Basal production of NO in the heart has been demonstrated in vascular endothelial cells as well as in cardiac myocytes by a constitutive endothelial NO synthase.[5,21,22] The reduction in coronary flow by L-NMMA or ODQ confirms that the basal production of NO, through activation of soluble guanylate cyclase, contributes to coronary vasodilator tone in the isolated heart.[5] Moreover, NO synthase has also been localized to mitochondria in the heart.[23] Approximately 1% of electron flow contributes to the formation of $O_2^{\bullet-}$ during normal mitochondrial respiration.[24] Nitric oxide and $O_2^{\bullet-}$ react at a diffusion-limited rate to form $ONOO^-$.[25] Endothelial cells simultaneously produce both NO and $O_2^{\bullet-}$[26] and agonist-stimulated production of $ONOO^-$ has also been shown in these cells.[27] A possible physiological role of $ONOO^-$ has been supported by studies showing that $ONOO^-$ can relax vascular smooth muscle,[28] inhibit platelet aggregation[10] and stimulate soluble guanylate cyclase.[9] These beneficial actions of $ONOO^-$, in contrast to its cytotoxic actions[25] following its protonation at physiological pH and decomposition to highly reactive intermediates,[29] are dependent upon the ability of $ONOO^-$ to react with thiols, in particular GSH, to form *S*-nitrosoglutathione.[9,10] Given the dependency upon NO and $O_2^{\bullet-}$ for the vasodilator effect of GSH, the data suggest that there is a significant basal

production of $ONOO^-$ in the isolated heart. Although the reaction efficiency of $ONOO^-$ and GSH to form *S*-nitrosoglutathione is about 1% in non-physiological solution,[9] recent evidence suggests that bicarbonate/CO_2 solution such as I used here can potentiate the reactivity of $ONOO^-$ (Yasmin and Schulz, unpublished observations).[30] Moreover, *S*-nitrosoglutathione is a potent NO donor active at nanomolar concentrations.[9,31] Peroxynitrite has also been shown to oxidize thiols to their disulfides,[32] however, *S*-nitrosation reactions were not examined at the time. Although oxidation of thiols is important to some of the toxic actions of $ONOO^-$, the balance between thiol oxidation and thiol nitrosation to form NO donors depends upon the thiol involved and should be examined under more physiological conditions (i.e. in bicarbonate/CO_2 solution).

Other mechanisms by which GSH can cause NO and soluble guanylate cyclase-dependent relaxation are possible. Glutathione can facilitate the dismutation of $O_2^{\bullet-}$ and thus reduce the amount of $O_2^{\bullet-}$. This prolongs the half-life of NO. Glutathione can also protect NO synthase from self inactivation by NO.[33] Ghigo et al demonstrated that both basal and Ca^{2+} ionophore-stimulated NO production in endothelial cells were increased in the presence of GSH.[20] They suggested that GSH was necessary for NO synthesis rather than for the effect of NO on guanylate cyclase. Moreover, GSH itself potentiates the formation of NO from *S*-nitrosoglutathione.[9]

The vascular effect of thiols can differ markedly, as shown in this study by the action of GSH in contrast to the complete lack of effect of L-cysteine. The vasodilator effect of GSH and its disulfide are consistent with a study by Fujioka et al which demonstrated concentration-dependent vasorelaxation with GSH and GSSG

(from 0.1 to 10 mM) in isolated dog coronary artery strips contracted with prostaglandin $F_{2\alpha}$.[7] These authors also showed that L-cysteine (from 0.1 to 10 mM), but not GSH or GSSG, caused a biphasic contraction in coronary arterial strips under resting tension conditions. However, in prostaglandin $F_{2\alpha}$ contracted strips, L-cysteine showed a modest vasorelaxant effect with lower efficacy than that of GSH or GSSG. Fujioka et al speculated that the vascular action of thiol compounds was due to either: a) their direct actions on smooth muscle cells through the reduction of disulfide bonds of receptors, channels or enzymes, b) the reduction of small molecular weight disulfides to sulfhydryl compounds, and/or, c) the production of nitrosothiols. The absence of an effect of L-cysteine in the intact coronary circulation could be due to a number of possibilities, including: a) the rapid autooxidation of L-cysteine,[34] b) the requirement for increased coronary vascular tone before being able to detect vasodilation, and c) the far greater instability of nitrosocysteine in aqueous systems or a lower reaction efficiency of its formation compared to nitrosoglutathione.

Caution is required in extrapolating results from the isolated heart model to both in vivo and human pathophysiological conditions. The lack of cells and other antioxidant components of blood may accentuate the effect of thiols on coronary vascular tone. However, the significance of GSH in biological systems cannot be underestimated.[1] Glutathione is likely an important endogenous buffer against oxidant stress also by its action as a scavenger of $ONOO^-$. Exogenous administration of GSH and related thiols have been used to enhance or supplement the antioxidant function of tissues and cells.[16] This could be of particular importance in experimental models and clinical situations, such as during ischemia and reperfusion, where the

enhanced production of NO and $ONOO^-$ has been shown to be detrimental to cardiac function.[35] The use of blood (which contains GSH in micromolar concentrations) to perfuse isolated hearts and the addition of GSH into cardioplegic solutions have been demonstrated to improve cardiac function.[36,37] In summary, I have demonstrated a concentration-dependent vasodilation in the intact coronary circulation with GSH and its disulfide which was related to the activation of a NO- and soluble guanylate cyclase-dependent mechanism, possibly mediated by the reaction between GSH and $ONOO^-$ to form *S*-nitrosoglutathione, a NO donor.

Table 2.1 The action of glutathione (GSH) and glutathione disulfide (GSSG) on heart rate (HR), left ventricular developed pressure (LVDP) and rate-pressure product (RPP) in isolated rat hearts compared with a time control group.

	time	HR (min^{-1})	LVDP (mmHg)	RPP ($x10^{-3}$ mmHg/min)
TIME CONTROL (n = 6)				
	0 min	245 ± 13	97 ± 3	24.0 ± 1.8
	10 min	250 ± 12	99 ± 3	24.7 ± 1.3
	20 min	250 ± 12	95 ± 3	23.7 ± 1.2
	30 min	253 ± 10	93 ± 4	23.3 ± 1.3
	40 min	260 ± 15	89 ± 5	23.1 ± 1.5
	50 min	265 ± 15	86 ± 5	22.7 ± 1.4
	65 min	260 ± 14	84 ± 8	21.6 ± 2.0
GSH (n = 7)				
Baseline	0 min	261 ± 9	96 ± 3	25.0 ± 0.9
GSH 10 µM	10 min	261 ± 10	94 ± 4	24.4 ± 0.9
GSH 30 µM	20 min	258 ± 10	97 ± 4	24.7 ± 0.9
GSH 100 µM	30 min	268 ± 8	89 ± 3	23.8 ± 0.7
GSH 300 µM	40 min	264 ± 7	84 ± 4	22.1 ± 0.8
GSH 1000 µM	50 min	254 ± 9	68 ± 4*	20.6 ± 0.9
Recovery	65 min	259 ± 9	79 ± 5	20.1 ± 0.9
GSSG (n = 6)				
Baseline	0 min	257 ± 8	95 ± 7	25.4 ± 1.9
GSSG 10 µM	10 min	263 ± 10	94 ± 7	25.5 ± 1.9
GSSG 30 µM	20 min	264 ± 10	93 ± 7	25.5 ± 2.0
GSSG 100 µM	30 min	266 ± 10	87 ± 6	24.1 ± 2.0
GSSG 300 µM	40 min	269 ± 12	79 ± 6	21.9 ± 1.8
GSSG 1000 µM	50 min	267 ± 11	68 ± 6*	19.1 ± 2.0
Recovery	65 min	271 ± 9	79 ± 8	19.0 ± 3.6

Values represent mean ± s.e.m, * $P < 0.05$ vs. respective value in the time control group (one-way ANOVA)

Table 2.2 No effect of glybenclamide (1 μM, n = 4) and indomethacin (4 μM, n = 3) on the vascular action of glutathione (GSH, 300 μM) or glutathione disulfide (GSSG, 300 μM) in isolated rat hearts.

	Coronary flow (ml/min)	
	GSH	GSSG
Baseline	13.3 ± 0.3	13.5 ± 0.3
Glybenclamide	12.7 ± 0.9	12.7 ± 0.3
Glybenclamide + GSH or GSSG	16.0 ± 1.0*	15.0 ± 0.6*
Baseline	13.7 ± 0.3	13.3 ± 0.3
Indomethacin	13.3 ± 0.3	12.7 ± 0.3
Indomethacin + GSH or GSSG	17.3 ± 0.9*	17.3 ± 0.3*

Values represent mean ± s.e.m., * $P < 0.05$ vs. respective baseline value (one-way repeated measures ANOVA)

Fig. 2.1 Vasodilator action of (A) glutathione (n = 7) and (B) glutathione disulfide (n = 6) in isolated rat hearts.

Increasing concentrations of glutathione or glutathione disulfide were infused at 10-min intervals and coronary flow was recorded at 1, 2, 5 and 10 min of infusion at each concentration. A 15 min recovery period after infusion of 1000 μM is also shown. Numbers in bar indicate concentration (μM).

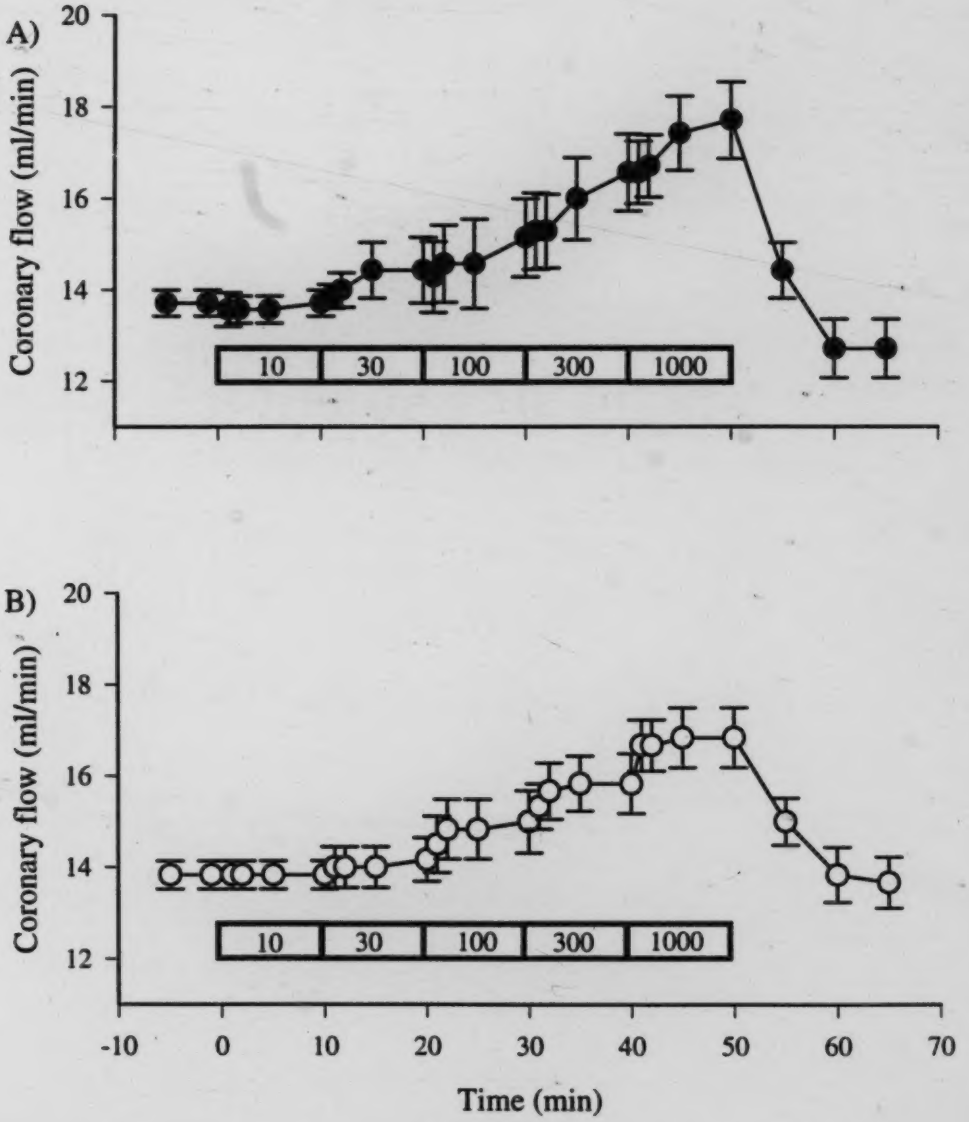


Fig. 2.2 Concentration-dependent vasodilator action of glutathione and related compounds on coronary flow.

Coronary flow following 10 min of infusion at each concentration of glutathione (GSH, 10-1000 μM, n = 7), glutathione disulfide (GSSG, 10-1000 μM, n = 6), glutathione ethyl ester (GSHee, 3-300 μM, n = 6) or L-cysteine (L-CYS, 10-1000 μM, n = 5) is plotted against concentration. C denotes baseline coronary flow values (control).

* $P < 0.05$ vs. respective control (one-way repeated measures ANOVA)

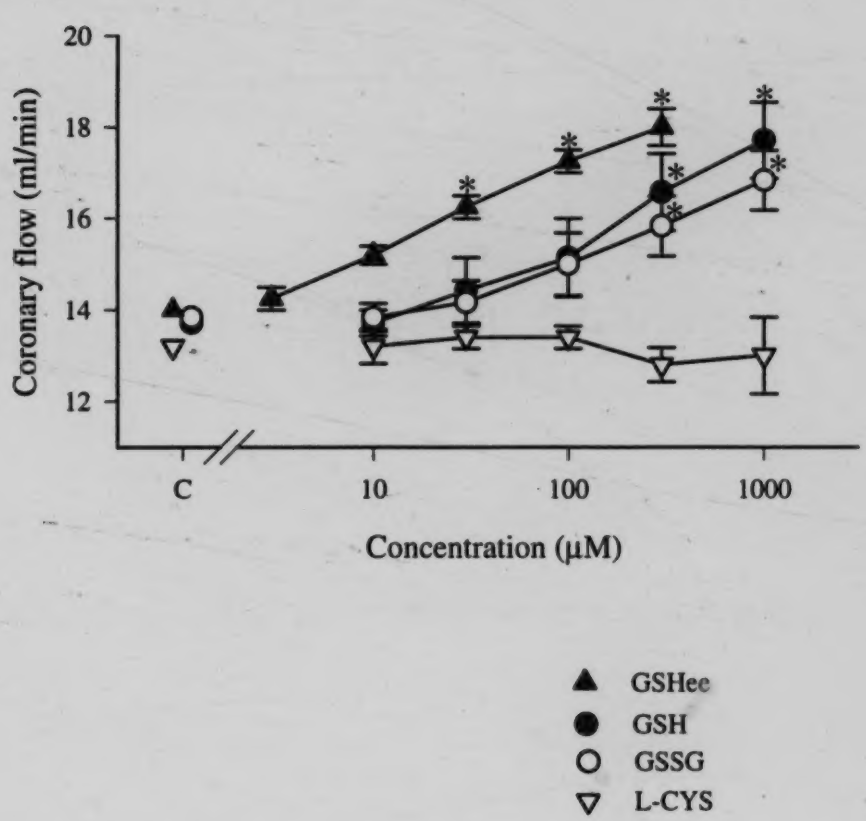


Fig. 2.3 L-NMMA inhibits the vasodilation with (A) glutathione (GSH) and (B) glutathione disulfide (GSSG).

C denotes baseline coronary flow values (control). n = 3-4 in each group.

* $P < 0.05$ (one-way ANOVA)

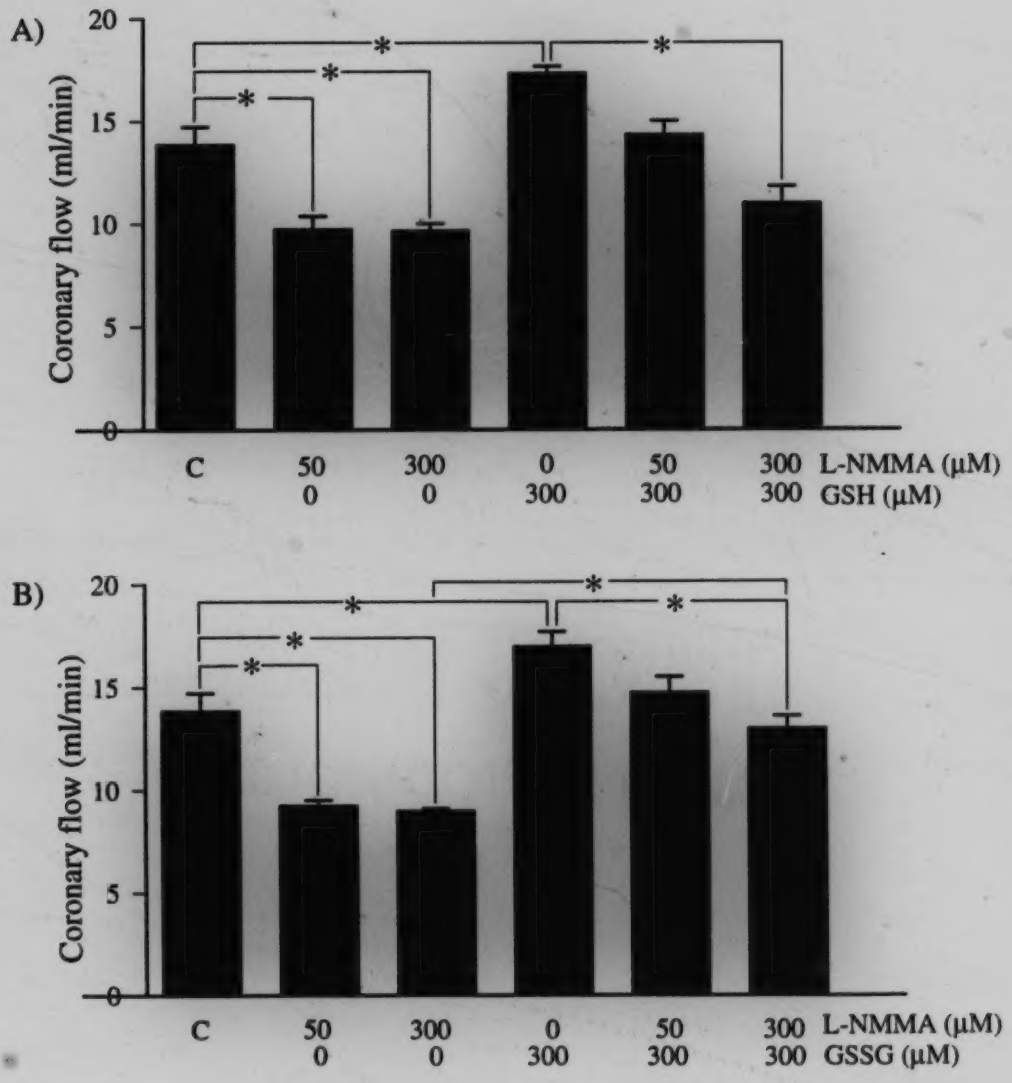


Fig. 2.4 The reversal of L-NMMA's inhibitory effect on (A) glutathione (GSH) or (B) glutathione disulfide (GSSG) mediated vasodilation with L-arginine (L-arg, open bars) but not D-arginine (D-arg, closed bars).

L-arg (1 mM) or D-arg (1 mM) were co-infused with GSH (300 μM) or GSSG (300 μM) during the final 15 min of a 30-min infusion of L-NMMA (300 μM). C denotes baseline coronary flow values (control). n = 3 in each group.

* P < 0.05 (one-way ANOVA)

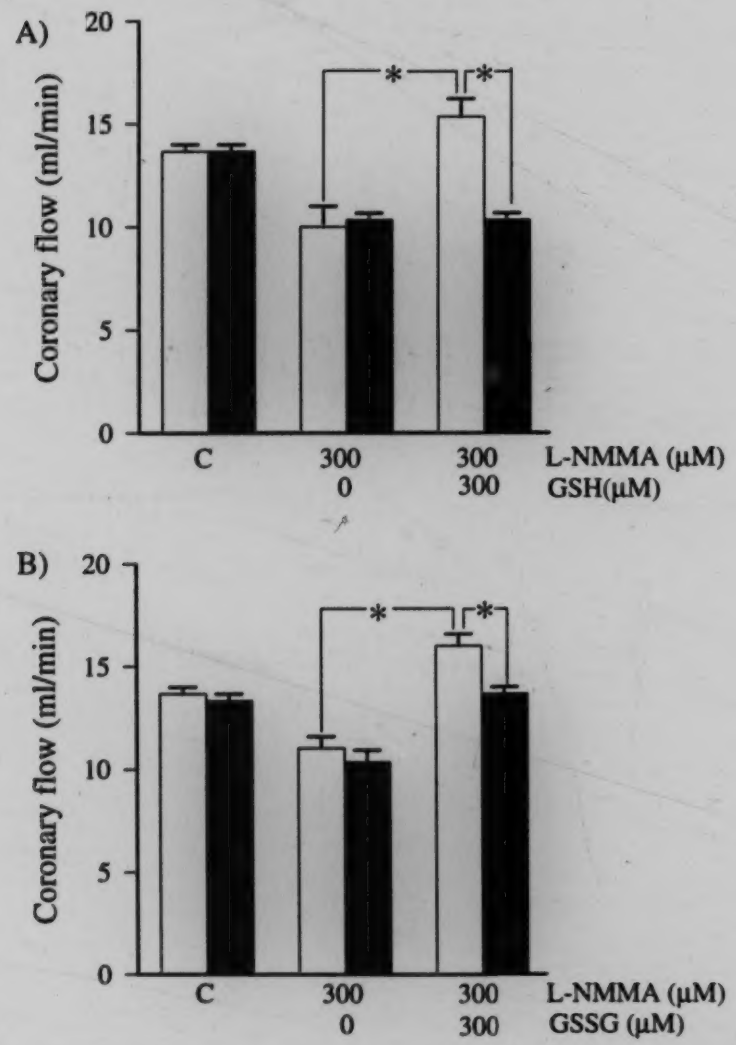


Fig. 2.5 ODQ inhibits the vasodilation with (A) glutathione (GSH, 300 μM) and (B) glutathione disulfide (GSSG, 300 μM).

C denotes baseline coronary flow values (control). n = 3-8 in each group.

* $P < 0.05$ (one-way ANOVA)

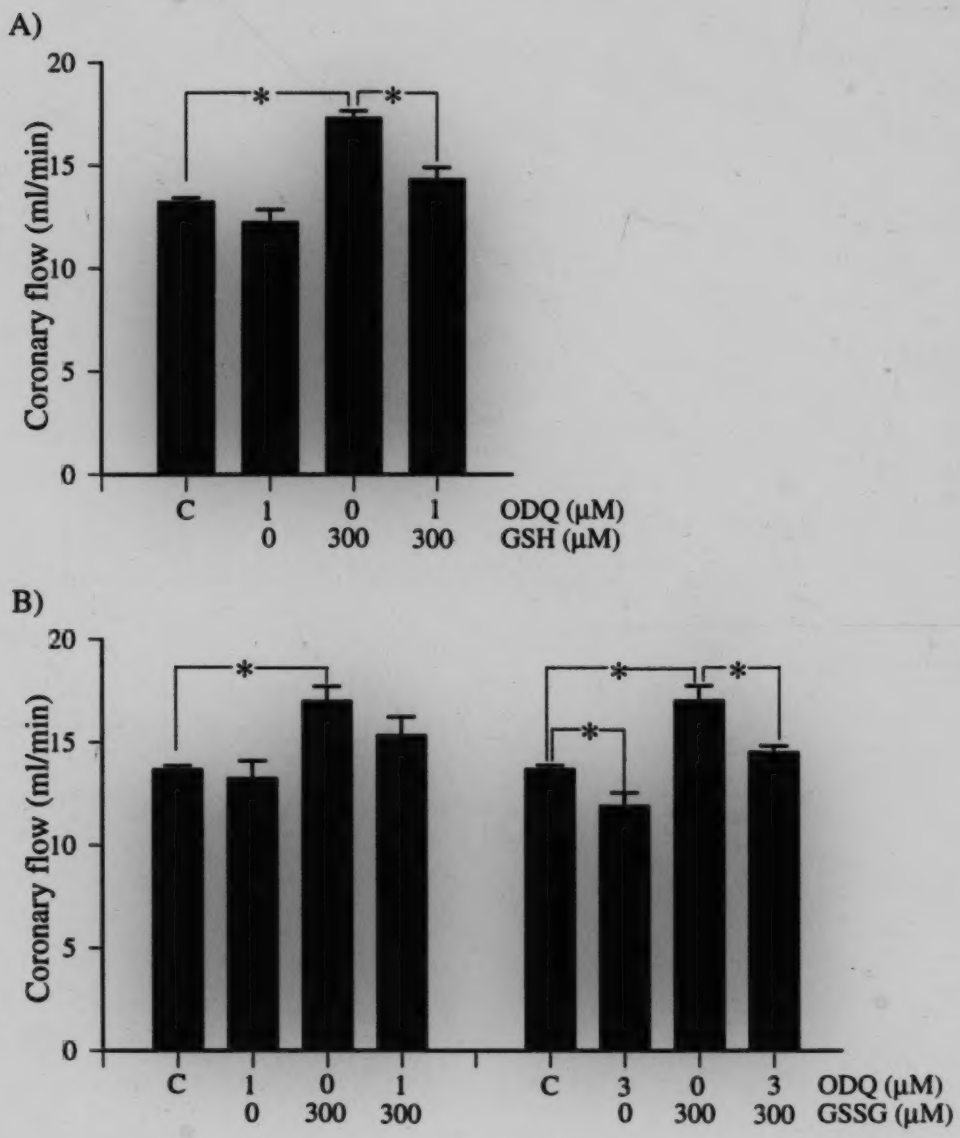


Fig. 2.6 ODQ inhibits the increase in myocardial cyclic GMP levels during infusion of (A) glutathione (GSH, 300 μM) or (B) glutathione disulfide (GSSG, 300 μM).

C denotes baseline cyclic GMP levels in time control hearts. n = 3 in each group.

* $P < 0.05$ (one-way ANOVA)

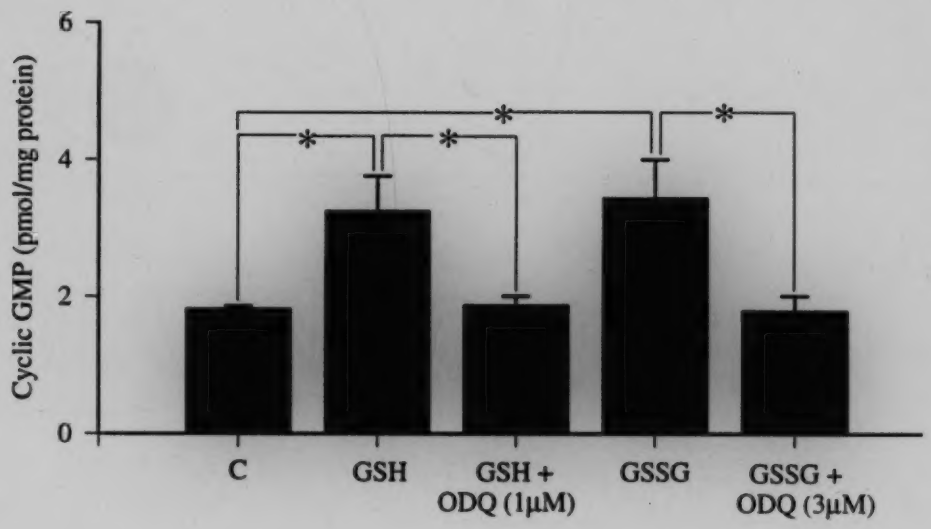


Fig. 2.7 Superoxide dismutase (SOD, 50 U/ml) inhibits the vasodilation with glutathione (GSH, 300 μM).

C denotes baseline coronary flow values (control). n = 4 in each group.

* P < 0.05 (one-way ANOVA)

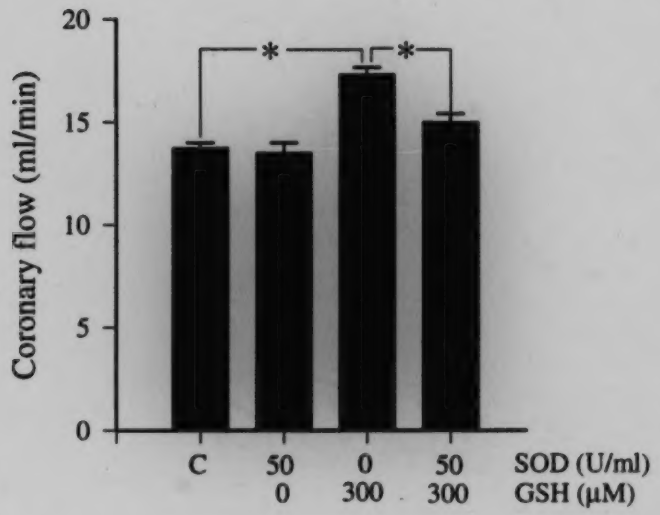
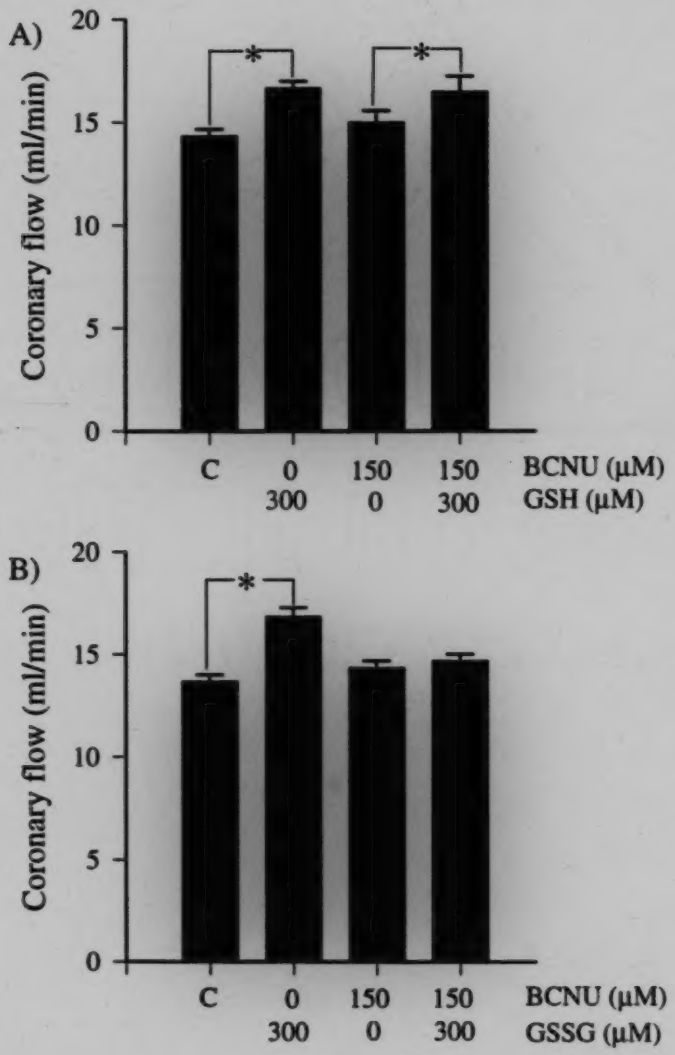


Fig. 2.8 The effect of the glutathione reductase inhibitor 1, 3-bis(2-chloroethyl-)-1-nitrosourea (BCNU, 150 μM) on vasodilation with (A) glutathione (GSH, 300 μM) and (B) glutathione disulfide (GSSG, 300 μM). BCNU was infused for 30 min. Five min following the discontinuation of BCNU, GSH or GSSG were infused for 10 min (see Methods for details).

C denotes equilibrium coronary flow values with vehicle control. n = 3 in each group.

* $P < 0.05$ (paired *t* test)



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CHAPTER III

THIOLS PROTECT AGAINST THE INHIBITION OF MYOCARDIAL ACONITASE BY PEROXYNITRITE

A version of this chapter has been published. Cheung P-Y, Danial H, Jong J, Schulz R. (1998) Arch Biochem Biophys 350:104-108.

ABSTRACT

Peroxynitrite ($ONOO^-$) is a potent inhibitor of myocardial aconitase. As $ONOO^-$ reacts with sulfhydryl moieties, the protective action of thiols against $ONOO^-$-mediated inhibition of aconitase was investigated. Aconitase activity was examined in ventricular homogenates prepared from freshly isolated rat hearts. Peroxynitrite, but not the nitric oxide donor S-nitroso-N-acetyl-*d,l*-penicillamine (0.03-300 μM), inhibited aconitase activity (IC_{50} = 47 ± 6 μM). L-cysteine (0.03-3 mM), glutathione (0.03-3 mM) and *N*-(2-mercaptoproprionyl)-glycine (MPG, 0.1-3 mM) protected against the inhibitory effect of $ONOO^-$ (100 μM) with the rank order of effectiveness of MPG > glutathione > L-cysteine. D-cysteine (3 mM) had a similar protective effect as L-cysteine, but L-cystine, the oxidized form of L-cysteine, offered no protection. Ferrous ammonium sulfate (1 mM) markedly enhanced the protection provided by L-cysteine, but not by glutathione or MPG. Thiols protect myocardial aconitase against inhibition by $ONOO^-$ in a manner which is sulfhydryl group dependent and not stereospecific. The protection is related to the maintenance of the redox state of the iron-sulphur cubane cluster and cysteine residues at the active site of the enzyme. Both naturally occurring thiols and thiol-based drugs may be useful to protect the heart during ischemia-reperfusion injury when there is an excessive production of $ONOO^-$.

INTRODUCTION

Aconitase is a citric acid cycle enzyme that converts citrate to isocitrate. It belongs to the family of iron-sulfur-containing dehydratases whose activities depend on the redox state of the cubane [4Fe-4S] cluster.[1,2] Cysteine residues around the iron cluster are also important for optimal activity.[3] Nitric oxide (NO) was originally reported to inactivate aconitase.[4] However, NO reacts with superoxide anion at a diffusion-limited rate to yield peroxynitrite ($ONOO^-$)[5] which is markedly more potent and efficacious than NO in inhibiting aconitase.[6,7] The endogenous formation of $ONOO^-$ contributes to myocardial ischemia-reperfusion injury[8] and has also been implicated in the pathogenesis of atherosclerosis,[9] septic shock[10] and neurodegenerative diseases.[11]

Endogenous thiols (glutathione, GSH; L-cysteine)[12] as well as thiol-containing agents such as *N*-(2-mercaptoproprionyl)-glycine (MPG)[13] are important antioxidants which protect cells and tissue from oxidative stress. Part of their antioxidant effect may be due to their ability to react with $ONOO^-$, forming disulfides[14] and nitrosothiol derivatives, such as *S*-nitrosoglutathione from the reaction of GSH and $ONOO^-$.[15,16] This detoxification of $ONOO^-$ may be a unique feature of this class of compounds, as this results in the formation of NO donors that antagonize the detrimental reactions of $ONOO^-$ in biological systems[8,16,17] or have cytoprotective actions on the cell through NO-mediated stimulation of soluble guanylate cyclase and other mechanisms.[18]

The action of a variety of thiols on $ONOO^-$-mediated inhibition of aconitase in rat heart homogenates was investigated. It was hypothesized that they would protect

myocardial aconitase activity from inhibition by $ONOO^-$ in a concentration-dependent fashion and that their action would depend upon a free sulfhydryl group.

MATERIALS AND METHODS

S-nitroso-N-acetyl-*d,l*-penicillamine (SNAP) was purchased from Alexis Corp (San Diego, CA, USA). Peroxynitrite and decomposed $ONOO^-$ (vehicle control) were synthesized as previously described.[8] The concentration of $ONOO^-$ was verified on the day of the experiment by ultraviolet spectroscopy.[8] All other reagents were purchased from Sigma (Mississauga, ON, Canada). Fluorocitrate and L-cystine were dissolved in 0.2 M HCl and 0.4 M NaOH, respectively, and all the other reagents were dissolved in deionized water.

Isolation of hearts and preparation of ventricular extract

Male Sprague-Dawley rats weighing 250-300 g were used. Following intraperitoneal pentobarbital overdose (60 mg/kg), hearts were rapidly excised and briefly rinsed by immersion into ice-cold Krebs-Henseleit solution. Then they were perfused via the aorta at a constant hydrostatic pressure of 60 mmHg with Krebs-Henseleit solution at 37 °C to wash them free of blood. The composition of the Krebs-Henseleit solution was (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), and EDTA (0.5) and it was continuously gassed with 95 % O_2 / 5 % CO_2 (pH 7.4). After 10 min of perfusion, the ventricles were freeze-clamped with tongs cooled to the temperature of liquid nitrogen. Frozen ventricles were pulverized under liquid nitrogen with a mortar and pestle. The frozen powdered tissue (200-250 mg wet weight) was suspended in 1 ml of ice-cold homogenization buffer consisting of 10 mM Hepes, 0.1 mM EDTA, 0.32 M sucrose, 1 mM *dl*-dithiothreitol, 10 µg/ml trypsin inhibitor, 10 µg/ml leupeptin and

2.1 mg protein/ml aprotinin (pH 7.4). The protease inhibitor phenylmethylsulfonyl fluoride was added to the suspension (100 µg/ml) which was then homogenized at 4 °C. The heart extracts were centrifuged (14,000 g, 5 min at 4 °C) and the supernatant was separated and kept on ice until analysis.

Experimental Procedures

The effect of $ONOO^-$ on myocardial aconitase activity was investigated by adding 5 µl of concentrated stock solutions of $ONOO^-$ as a single bolus to a mixture of the ventricular extract (50 µl) and 100 mM Tris HCl buffer (200 µl, pH 7.4), contained in light-protected microvials (#TECO50-N from DiaMed Lab Supplies Inc., Mississauga, ON, Canada) to give a final concentration between 0.1-1000 µM. The reaction mixture was incubated for 5 min at 21°C. Aconitase activity of an aliquot of the reaction mixture was then measured as described below. The actions of 5 µl of decomposed $ONOO^-$ (vehicle control, 0.1-1000 µM equivalent) or SNAP (0.03-300 µM) were assessed in an identical manner.

In order to determine the nature of the inhibitory effect of $ONOO^-$ on aconitase, experiments were performed with the suicide substrate inhibitor of aconitase, fluorocitrate at either high (3 mM) or low (10 µM) concentration.[19] Fluorocitrate (5 µl) was added to give 3 mM final concentration to the ventricular extract in Tris HCl buffer (250 µl), incubated for 5 min at 21°C and aconitase activity of the reaction mixture was then measured. The effect of 10 µM fluorocitrate on $ONOO^-$-mediated inhibition of aconitase was evaluated by a 5 min pre-incubation of

the ventricular extract in Tris HCl buffer (250 µl) with fluorocitrate (5 µl) followed by 5 min at 21°C with 300 µM $ONOO^-$ (5 µl).

The concentration-dependent effects of GSH (30-3000 µM), L-cysteine (30-3000 µM), D-cysteine (3000 µM), MPG (100-3000 µM) and L-cystine (3000 µM in 0.4 M NaOH) were evaluated by a 5 min pre-incubation at 21°C of the ventricular extract in Tris HCl buffer (250 µl) with the studied compound (5 µl) followed by 5 min at 21°C with 100 µM $ONOO^-$ (5 µl). Control experiments to determine the direct effect of the thiols on aconitase activity (in the absence of $ONOO^-$) were also performed. None of the reagents caused a significant alteration in the pH of the reaction mixture.

The effect of ferrous ammonium sulfate (5 µl) on $ONOO^-$-mediated aconitase inhibition was studied by a 5 min pre-incubation at 21°C of the ventricular extract in Tris HCl buffer (250 µl) with the ferrous compound (0.1-1 mM) followed by 5 min at 21 °C with 100 µM $ONOO^-$ (5 µl). The direct effect of ferrous ammonium sulfate on aconitase activity was evaluated in a similar fashion in the absence of $ONOO^-$. Experiments to determine the effect of ferrous ammonium sulfate (1 mM) on the action of the thiols (3 mM) on $ONOO^-$-mediated aconitase inhibition were also performed by a 5 min pre-incubation at 21 °C of the ventricular extract in Tris HCl buffer (250 µl) with the ferrous compound (5 µl) and the studied thiol (5 µl) followed by 5 min at 21 °C with 100 µM $ONOO^-$ (5 µl).

Aconitase Activity Assay

The aconitase activity in the reaction mixture was measured in triplicate for 10 min at 21°C by a coupled microplate assay using isocitrate dehydrogenase, in which $NADP^+$ reduction is measured, as described by Rose and O'Connell.[20] Briefly, a 50 µl aliquot of the reaction mixture was added to 200 µl aconitase assay buffer consisting of 100 µl citrate concentrate (100 mM in 3 mM magnesium chloride and 100 mM Tris-HCl, pH 7.4), $NADP^+$ (2 µl, 60 mM), isocitrate dehydrogenase (2 µl, 125 units/ml in 50 % glycerol) and deionized water (96 µl). The rate of $NADP^+$ reduction was measured spectrophotometrically at 340 nm over 10 min at 21 °C. Protein content of the ventricular extracts was assessed using bicinchoninic acid reagent and bovine serum albumin as a standard. The enzyme activity was calculated and expressed in mU/mg protein, where one unit of activity is defined as the amount of enzyme necessary to produce 1 µmol *cis*-aconitate/min. The actions of the test thiols on aconitase activity were compared with the baseline activity of ventricular extract from the same heart sample and were expressed as a percentage of baseline.

STATISTICAL ANALYSIS

All experiments were performed in triplicate and were repeated using a minimum of three separate ventricular extracts. Data are expressed as means ± standard error and were analyzed by one-way ANOVA using Jandel SigmaStat 2.0 statistical software (Jandel Corporation, San Rafael, CA, USA). Post-hoc testing was performed using Fisher's least significant difference method. P value < 0.05 was considered to indicate statistical significance.

RESULTS

1. Effects of $ONOO^-$ and SNAP on aconitase activity

$ONOO^-$ (0.1-1000 μM) caused a concentration-dependent inhibition of aconitase activity with an IC_{50} of 47 ± 6 μM (n = 4, Fig. 3.1). The threshold inhibitory effect occurred at 10 μM $ONOO^-$; at 1000 μM $ONOO^-$ aconitase activity was abolished. In marked contrast, the equivalent vehicle strength of decomposed $ONOO^-$ or the NO donor, SNAP (0.03-300 μM) had no effect on aconitase activity (Fig. 3.1).

2. Protection from $ONOO^-$-mediated inhibition of aconitase using fluorocitrate

At high concentration, fluorocitrate (3 mM) abolished aconitase activity (to 5 ± 1% of control, n = 4). In contrast, 10 μM fluorocitrate, which in itself reduced aconitase activity to 70 ± 3%, significantly protected from the inhibitory effect of 300 μM $ONOO^-$ (3 ± 1% and 65 ± 4% in the absence or presence of fluorocitrate, respectively, $P < 0.05$, n = 6).

3. Concentration-dependent action of thiols against $ONOO^-$-mediated inhibition of aconitase activity

Each of the thiols tested, GSH (30-3000 μM), L-cysteine (30-3000 μM) and MPG (100-3000 μM), showed significant concentration-dependent protective actions against the inhibition of aconitase activity by 100 μM $ONOO^-$ (Fig. 3.2). The maximum protective effects of GSH, L-cysteine and MPG (58 ± 2%, 55 ± 3% and 62

± 2%, respectively) were not significantly different. Apparent EC_{50} values of the thiols were 0.27 ± 0.09 mM, 0.43 ± 0.16 mM and 0.80 ± 0.12 mM for MPG, GSH and L-cysteine, respectively (n = 4-5 in each group). The apparent EC_{50} of MPG was significantly lower than that of L-cysteine. At the highest concentration tested, 3 mM L-cysteine, but not GSH or MPG, had a slight inhibitory effect on aconitase activity (82 ± 2%, $P < 0.05$ vs. control) (Fig. 3.2).

4. Comparison between L-cysteine, D-cysteine and L-cystine on $ONOO^-$-mediated inhibition of aconitase

L-cystine, the oxidized form of L-cysteine lacking a free sulfhydryl group, did not protect the $ONOO^-$-mediated inhibition of aconitase (Fig. 3.3). In contrast, D-cysteine (3 mM) had a similar protective effect as 3 mM L-cysteine (Fig. 3.3).

5. Enhancement of protective effect of thiols by ferrous ammonium sulfate

Ferrous ammonium sulfate showed a significant inhibitory effect on aconitase activity at 0.1 and 0.5 mM but not at 1 mM (Table 1). In contrast, it reduced $ONOO^-$-mediated inhibition of aconitase at 1 mM but not at lower concentrations (Table 1). In the presence of 1 mM ferrous ammonium sulfate, the protective effect of L-cysteine, but not that of GSH or MPG, against $ONOO^-$-mediated inhibition of aconitase, was enhanced (Fig. 3.4).

DISCUSSION

In this study, $ONOO^-$-mediated inhibition of aconitase activity could be protected by the thiols GSH, L-cysteine and MPG, in a concentration-dependent manner. Each of the thiols had similar efficacy but different effectiveness in their effect. This protection requires the presence of free sulfhydryl group and is not stereospecific. Hausladen et al[6] and Castro et al[7] demonstrated that $ONOO^-$, but not NO, inhibited aconitase from *Escherichia Coli* homogenate and porcine heart mitochondria, respectively. We confirmed that myocardial aconitase was inhibited by $ONOO^-$, but not by NO released from SNAP. Despite the formation of nitrosyl-iron complexes which is expected at high NO (> 100 μM) levels and leads to moderate degrees of reversible inhibition of aconitase,[7] it is unlikely that physiological concentrations of NO could inhibit the aconitase activity in the absence of superoxide.

At millimolar concentrations, the suicide substrate inhibitor fluorocitrate inactivates aconitase by forming a covalent bond with the active site.[19] The protective effect of a low concentration of fluorocitrate (10 μM) suggests that both fluorocitrate and $ONOO^-$ share a common site of action because the resultant aconitase activity following treatment with 10 μM fluorocitrate and 300 μM $ONOO^-$ was similar to that of fluorocitrate alone and much greater than that following $ONOO^-$. The activity of aconitase depends on the redox condition of the cubane [4Fe-4S] cluster and the surrounding cysteine residues.[1-3] The activation and inactivation of the [4Fe-4S] cluster is a dynamic cyclical process that is modulated by superoxide anion and intracellular thiols.[21] These effects are explained by oxidation of susceptible [4Fe-4S] clusters with concomitant loss of Fe(II) by pro-oxidant species. Therefore, I speculate

that $ONOO^-$ exerts its inhibitory action by oxidizing the cubane [4Fe-4S] cluster as well as the cysteine residues around the cluster (Fig. 3.5).

The intracellular concentration of GSH is 5 – 10 mM[12] whereas that of cysteine is 10-100 μM.[22] The apparent EC_{50} values of GSH and L-cysteine on $ONOO^-$-mediated inhibition of aconitase were 0.43 mM and 0.80 mM, respectively. Hence, I speculate that GSH rather than L-cysteine plays an important physiological role in vivo in the protection of enzyme activity from oxidative stress. In addition, GSH is likely to be important for the transport and catabolism of NO under aerobic conditions.[23] GSH is a unique scavenger of $ONOO^-$ resulting in the formation of disulfides and *S*-nitrosoglutathione.[14-16] *S*-nitrosoglutathione can release NO that antagonizes the detrimental effects of $ONOO^-$.[8,16,17] There are other possible mechanisms accountable for the protective action of thiols. Thiols may also protect the inhibition of aconitase by maintaining a reduced Fe(II) state in the cubane [4Fe-4S] cluster. This is supported by the fact that the protection was free sulfhydryl group dependent and not stereospecific. However, the protective effect of thiols can also be related to a thiol-disulfide exchange. This thiol-disulfide exchange action of reduced thiols has been shown to be important in the activity of other enzymes containing cysteine residues.[24] Consistent with the report by Castro et al,[7] we observed that thiols or ferrous iron partially prevented the inhibition of aconitase by $ONOO^-$. This further suggests that $ONOO^-$ has multiple inhibitory actions on aconitase, including the loss of Fe(II) from the [4Fe-4S] cluster.

The concentration-dependent effect of ferrous ammonium sulfate on aconitase activity may depend on the balance between free oxygen radical generation catalyzed

by Fe^{2+} (via the Fenton reaction) and the maintenance of a reduced Fe(II) state of the cubane [4Fe-4S] cluster. The results suggest that low concentrations of Fe^{2+} on its own enhances the generation of oxygen free radicals and inhibits aconitase, whereas at high concentration (1 mM), it protects aconitase by maintaining a reduced Fe(II) state (Table 1). This may explain why high concentrations of ferrous ammonium sulfate are necessary for the reactivation of aconitase in biological samples.[7] An enhanced protective action against $ONOO^-$ of thiols with ferrous ammonium sulfate was only observed with L-cysteine, but not with GSH or MPG. This indicates a unique action of ferrous ion and cysteine leading to more efficacious maintenance of redox potential and scavenging of $ONOO^-$. Dinitrosyl-iron complexes can be generated more effectively with GSH, possibly MPG as well, than with cysteine.[25] The resultant lower availability of GSH and MPG may explain their lack of enhancement of the protective action of ferrous ion.

Yasmin et al have recently shown that $ONOO^-$ is formed during the acute reperfusion of ischemic rat hearts and contributes to the impaired recovery of mechanical function.[8] The inhibition of myocardial aconitase by $ONOO^-$ following myocardial ischemia-reperfusion may play a role in the impaired myocardial function during reperfusion. Li et al have shown a fatal dilated cardiomyopathy accompanied by a marked inhibition of myocardial aconitase activity in mutant mice lacking manganese superoxide dismutase,[26] a condition where the endogenous production of $ONOO^-$ in the heart may be enhanced. However, infusion of $ONOO^-$, but not SNAP, into aerobically perfused, isolated working hearts caused a delayed depression of cardiac mechanical function that was not associated with any changes in oxygen

consumption.[27] While aconitase activity and oxygen consumption are not equivalent, the difference between the current findings and those in the isolated heart perfusion may be due to the lower bioavailability of $ONOO^-$ to mitochondria in myocardial cells in the latter model. With the administration of $ONOO^-$ to isolated perfused hearts, $ONOO^-$ needs to diffuse across several layers of biological membrane before it reaches mitochondria in myocardial cells, meanwhile, it may react with the sulfhydryl containing residues of the biological membrane. In addition, intact GSH reductase-peroxidase system in isolated perfused hearts may also decrease the reactivity and the bioavailability of $ONOO^-$ to mitochondria in myocardial cells.

This study suggests that exogenous thiols may be useful to protect the heart against $ONOO^-$-mediated oxidative reactions in pathological situations where there is excessive production of $ONOO^-$ such as ischemia-reperfusion. Development of thiol-based drugs with enhanced efficiency, potency and bioavailability will be useful to combat pathological conditions where $ONOO^-$ production is enhanced.

Table 3.1 The action of ferrous ammonium sulfate (Fe^{2+}) on myocardial aconitase activity in the absence and presence of peroxynitrite ($ONOO^-$).

Condition	Aconitase activity (% of baseline)	
	without $ONOO^-$	100 μM $ONOO^-$
Control	100	7.3 ± 0.9
Fe^{2+} 0.1 mM	48.8 ± 2.1*	8.4 ± 1.4
0.5 mM	56.8 ± 1.3*	15.7 ± 2.8
1 mM	93.8 ± 3.6	38.0 ± 6.8*

Ferrous ammonium sulfate was incubated with the ventricular extract in Tris HCl buffer for 5 min at 21 °C prior to reaction with $ONOO^-$ for 5 min.

* $P < 0.05$ vs. respectively controls, n = 3 in each group.

Fig. 3.1 Concentration-dependent inhibition of myocardial aconitase activity by peroxynitrite ($ONOO^-$, n = 4), but not by decomposed $ONOO^-$ (n = 4) and S-nitroso-N-acetyl-*d,l*-penicillamine (SNAP, n = 4-6).

* $P < 0.05$ vs. baseline activity

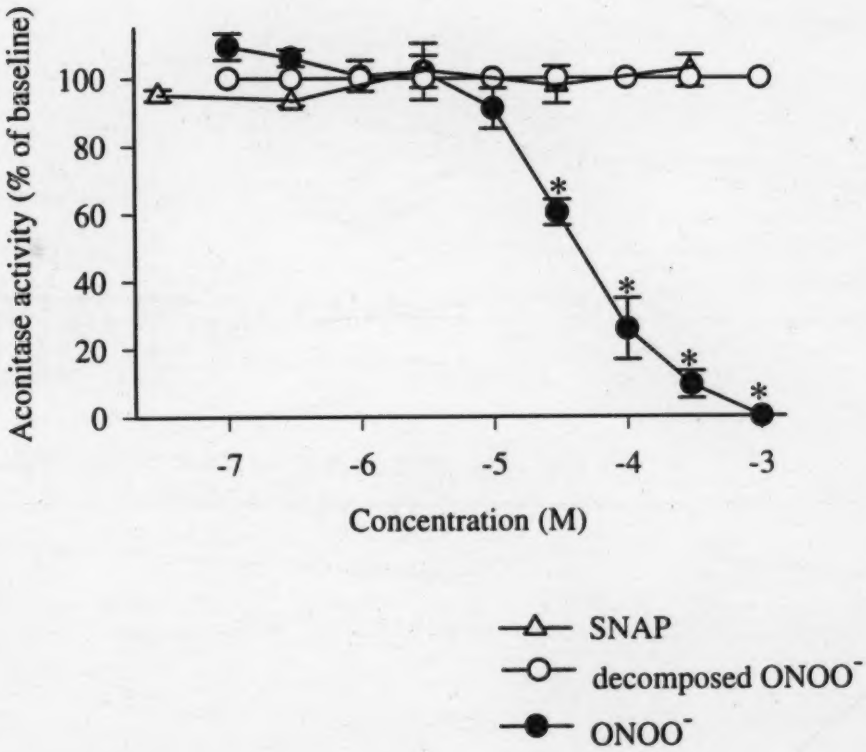


Fig. 3.2 Concentration-dependent protection of thiols (glutathione, GSH; L-cysteine, L-cys; and *N*-(2-mercaptoproprionyl)-glycine, MPG) against peroxynitrite ($ONOO^-$)-mediated inhibition of myocardial aconitase activity.

C = control activity. n = 4-5 in each group.

# $P < 0.05$ vs. control

* $P < 0.05$ vs. $ONOO^-$

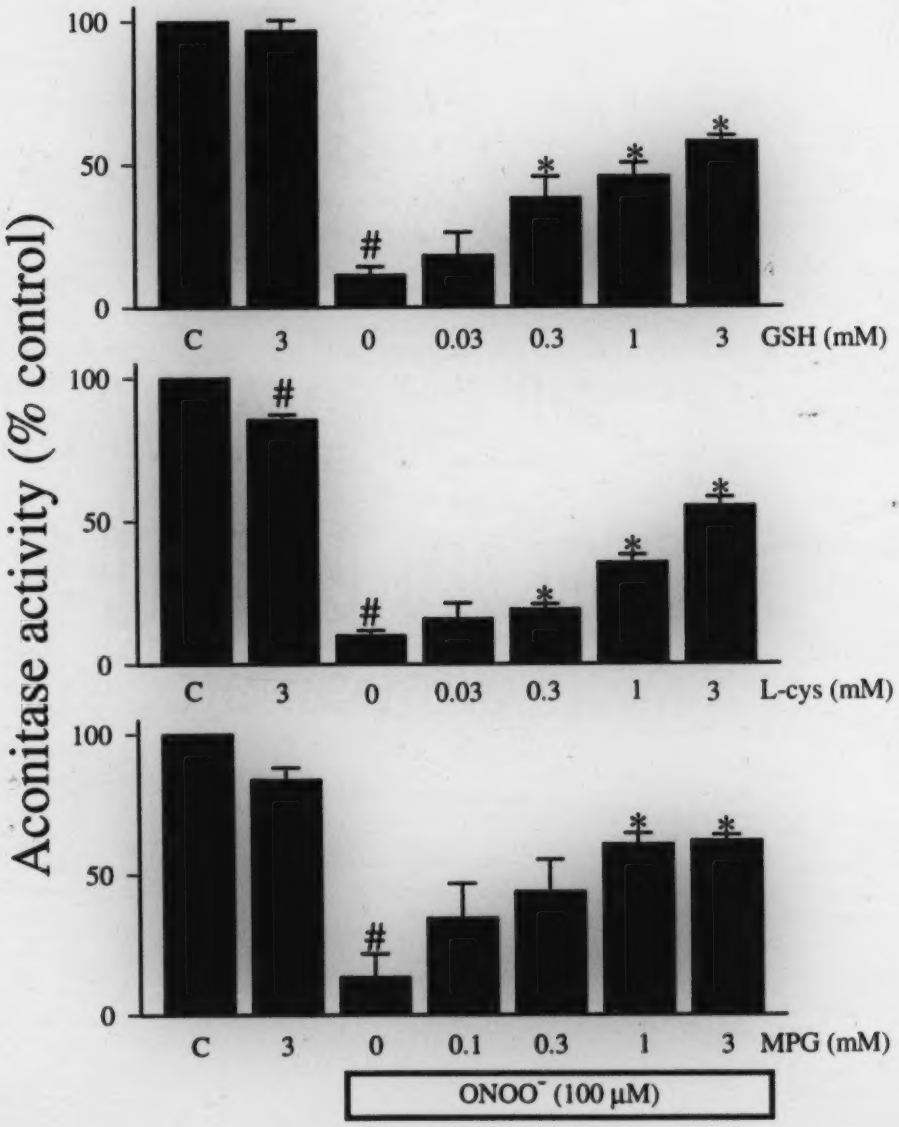


Fig. 3.3 L-cysteine (L-cys) and D-cysteine (D-cys), but not L-cystine (cys-cys) protect against peroxynitrite ($ONOO^-$)-mediated inhibition of myocardial aconitase activity.

C = control activity. n = 3 in each group.

# $P < 0.05$ vs. control

* $P < 0.05$ vs. $ONOO^-$

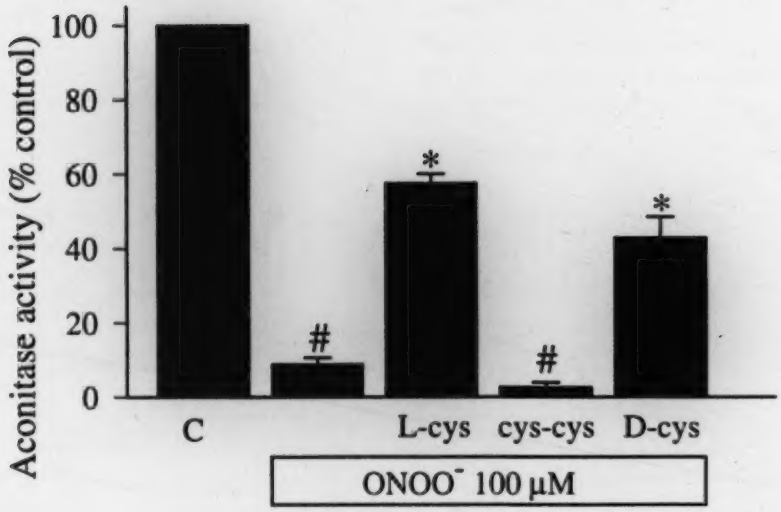


Fig. 3.4 The action of ferrous ammonium sulfate (Fe^{2+}, 1 mM) on the protection of L-cysteine (L-cys, 3 mM), glutathione (GSH, 3 mM) and *N*-(2-mercaptoproprionyl)-glycine (MPG, 3 mM) on peroxynitrite ($ONOO^-$, 100 μM)-mediated inhibition of myocardial aconitase activity.

C = control activity. n = 3 in each group.

# $P < 0.05$ vs. control

* $P < 0.05$ vs. $ONOO^-$

† $P < 0.05$ vs. $ONOO^-$ and L-cys

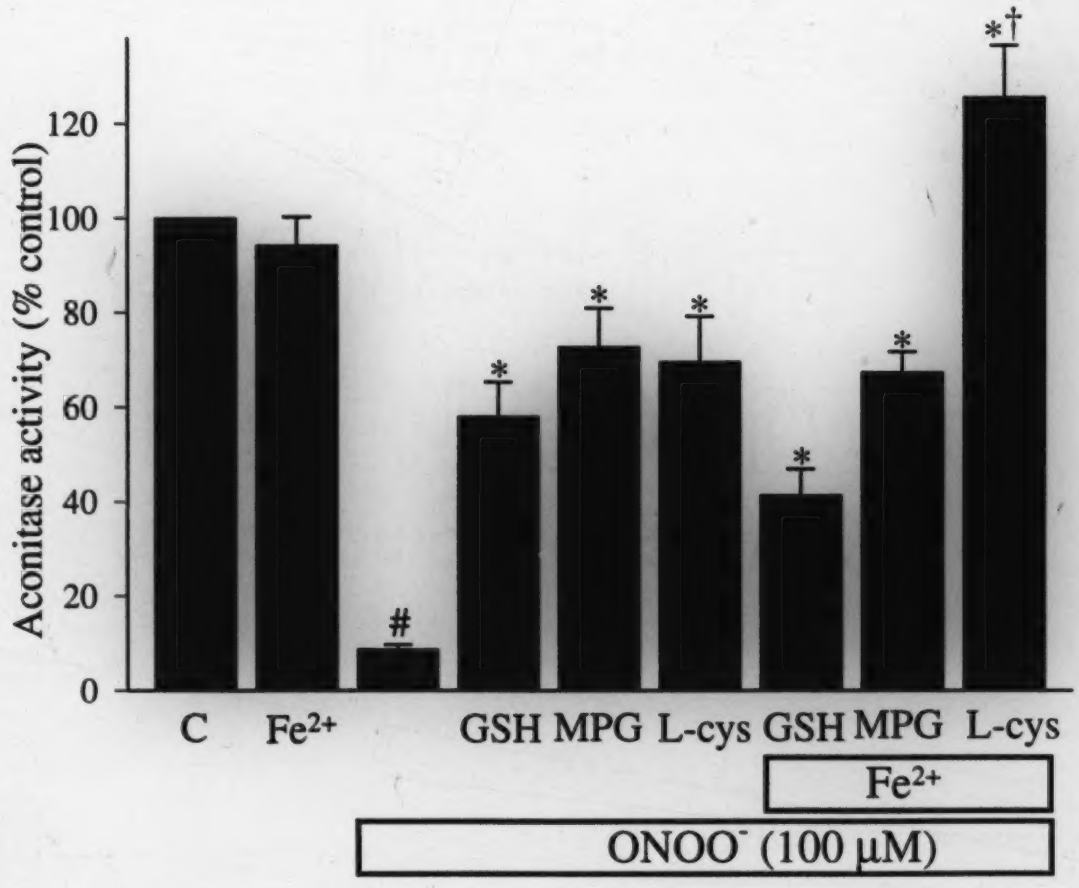
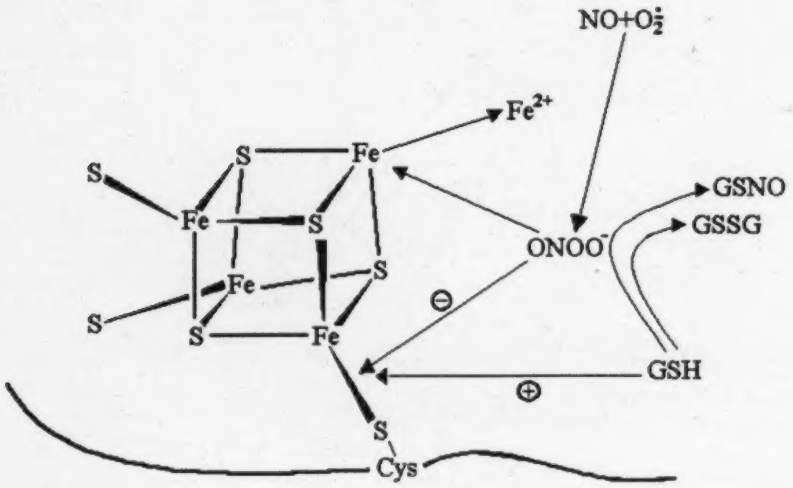


Fig. 3.5 Putative mechanism of inhibition on aconitase induced by peroxynitrite ($ONOO^-$).

Peroxynitrite may exert its inhibitory action by oxidizing the cubane [4Fe-4S] cluster as well as the cysteine residues around the cluster at the active enzymatic site. Curved line denotes the peptide chain of aconitase with cysteine residues located at 437, 503 or 506 position of the amino acids sequence.



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CHAPTER IV

GLUTATHIONE PROTECTS AGAINST MYOCARDIAL ISCHEMIA-REPERFUSION INJURY BY SCAVENGING PEROXYNITRITE

A version of this chapter has been submitted for publication. Cheung P-Y, Schulz R. (1998) Am J Physiol.

ABSTRACT

Peroxynitrite ($ONOO^-$) formation during acute reperfusion of the ischemic heart contributes to the poor recovery of mechanical function. As glutathione (GSH) scavenges $ONOO^-$, I tested the effect of GSH on the functional recovery of isolated rat hearts subjected to ischemia-reperfusion. Hearts were subjected to 25 min aerobic perfusion, 20 min global, no-flow ischemia and 30 min reperfusion. Glutathione (3-300 μM), GSH mono-ethyl ester (3 μM) or saline vehicle (control) were infused for 10 min prior to ischemia and throughout reperfusion. During reperfusion, GSH caused a concentration-dependent improvement in the recovery of mechanical function (as shown by rate-pressure product) which was significant at $\geq$ 30 μM GSH and was not associated with significant changes in the total concentration of GSH. Glutathione mono-ethyl ester (3 μM) mimicked the protective effect of 30 μM GSH. The concentration of dityrosine in the coronary effluent, a marker of $ONOO^-$ formation, was significantly reduced in hearts treated with 30 and 300 μM GSH and 3 μM GSH mono-ethyl ester, but not 3 μM GSH. Myocardial cyclic GMP levels were significantly elevated by 300 μM GSH during ischemia and early reperfusion. The mechanism by which GSH improves the recovery of myocardial mechanical function after ischemia-reperfusion may be related to its reaction with $ONOO^-$ to form *S*-nitrosoglutathione, a nitric oxide donor that stimulates soluble guanylate cyclase.

INTRODUCTION

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which forms the largest pool of non-protein thiols in the body[1] and is also the major antioxidant in the cell. In the presence of oxidant stress GSH is oxidized to glutathione disulfide by GSH peroxidase.[2] Glutathione is then regenerated from glutathione disulfide by GSH reductase. This redox cycle is important for the cell to combat oxidant stress.[2] Indeed, the increased capacity and turnover rate of the GSH redox cycle has been postulated to be the primary mechanism for the antioxidant function of GSH.[3] While hearts depleted of GSH have a greater susceptibility to ischemia-reperfusion injury,[4] the effect of exogenous GSH on recovery of myocardial function following ischemia-reperfusion or hypoxia-reoxygenation remains controversial.[5,6] Tani did not find any protective action of thiols such as GSH (2-10 mM), cysteine (1-2 mM), and N-acetylcysteine (0.002-2 mM) in myocardial ischemia-reperfusion injury in isolated rat hearts.[5] In contrast, Seiler et al demonstrated cardioprotective effects of GSH (1 or 10 mM) or GSH mono-ethyl ester (10 mM) in isolated rat hearts subjected to intermittent periods of hypoxia.[6] The mechanism by which GSH may mediate its protective effects in the heart is unclear.

I have reported a rapid coronary vasodilation in response to the infusion of GSH in micromolar concentrations in the aerobically perfused rat heart and suggested that GSH relaxes the coronary vasculature via a nitric oxide (NO) and soluble guanylate cyclase-dependent mechanism.[7] I speculated that GSH may act as a vasodilator by its reaction with peroxynitrite ($ONOO^-$), the reaction product of NO and superoxide ($O_2^{\bullet-}$) which may be generated at low levels during aerobic perfusion.[7]

This results in the production of *S*-nitrosoglutathione which then acts as a NO donor and mediates vasodilation via the stimulation of soluble guanylate cyclase.[8]

During the first min of reperfusion of the ischemic heart there is a burst of $ONOO^-$ production that contributes to the impairment of myocardial function.[9] The production of $ONOO^-$ and its detrimental effects in the heart are blocked by low concentrations of inhibitors of NO synthase.[9,10] In addition, infusion of a nitrosothiol which is a NO donor, S-nitroso-N-acetyl-d,l-penicillamine, also protected the heart, likely due to the ability of NO to interfere with the oxidative reactions of endogenously formed $ONOO^-$.[9,11,12,13] The ability of a variety of thiols including GSH to protect against $ONOO^-$-induced inhibition of metabolic enzyme activity was recently shown in rat heart homogenates.[14] I hypothesized that GSH acts as a unique scavenger of $ONOO^-$ through the formation of a NO donor intermediate, characterized by the stimulation of soluble guanylate cyclase, and improves the recovery of mechanical function during reperfusion following ischemic injury in isolated rat hearts.

MATERIALS AND METHODS

This investigation conforms with the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993).

Materials:

Glutathione, GSH mono-ethyl ester and L-tyrosine were purchased from Sigma Chemical Company. Latex balloons (size 4) were obtained from Radnoti Glass Technology (Monrovia CA, USA). Dityrosine was synthesized[15] and its purity was verified by high performance liquid chromatography.[16]

Heart Preparation and Perfusions

Male Sprague-Dawley rats (250-300 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). Hearts were rapidly excised and briefly rinsed by immersion in ice-cold Krebs-Henseleit solution. They were perfused via the aorta at a constant pressure of 60 mmHg with Krebs-Henseleit solution at 37 °C. The composition of Krebs-Henseleit solution was (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), EDTA (0.5) and L-tyrosine (0.3) and it was continuously gassed with 95 % O_2 / 5 % CO_2 (pH 7.4). L-tyrosine reacts with $ONOO^-$ to form the fluorescent product dityrosine.[17] Yasmin et al have previously shown that the concentration of dityrosine in the coronary effluent of isolated hearts perfused with Krebs-Henseleit solution containing L-tyrosine can be used as an index of $ONOO^-$ production by the heart .[9]

Spontaneously beating hearts were used in all experiments. A small latex balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve. The balloon was filled with deionized water (0.3-0.5 ml) to achieve a left ventricular end diastolic pressure of 8-12 mmHg. The transducer was connected to a Grass VI Polygraph on which heart rate and left ventricular pressure were monitored. Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricle. The rate-pressure product (RPP) was calculated as the product of heart rate and LVDP, and used as a measurement of cardiac mechanical function. Coronary flow was measured by an in-line ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) positioned proximal to the perfusion cannula. A water-jacketed glass chamber was positioned around the heart to maintain its temperature at 37 °C.

The intervals between thoracotomy and attachment of the heart to the perfusion system and between thoracotomy and beginning of stabilization period were less than 1 and 5 min, respectively. Using this perfusion protocol, hearts maintained a steady state of coronary flow, heart rate and LVDP for at least 80 min after stabilization.[7]

Experimental protocol

Following 15 min of stabilization, either GSH or saline vehicle solutions were infused for 10 min into the aerobically perfused heart via a side-port 15 cm proximal to the aortic cannula. The solutions were infused at a constant rate of 0.1 ml/min by a

syringe pump (Sage Instruments, Cambridge, MA, USA) and the concentration of the stock solutions was prepared based on an average coronary flow of 14 ml/min. Then 20 min of global, no-flow ischemia was induced by clamping the aortic inflow line and this was followed by 30 min of aerobic reperfusion subsequent to reopening the clamp. Coronary flow, heart rate, LVDP and RPP were determined at 15 and 25 min of aerobic perfusion prior to ischemia and at 5, 10, 20, and 30 min of the reperfusion period. Samples of coronary effluent were collected for the determination of the concentration of dityrosine as a marker of $ONOO^-$ generation at 15 and 25 min during aerobic perfusion and at 30-sec intervals for the first 2 min and then at 5 and 30 min of reperfusion. The samples were placed on ice and dityrosine fluorescence was measured within 15 min after sample collection as described.[9] The detection limit for dityrosine was 0.05 μM. The fluorescent intensity of dityrosine in the coronary effluent samples was expressed as a percentage of that seen at 15 min of aerobic perfusion in the same heart. Furthermore, fluorescence was also measured in solutions of authentic dityrosine (0.1-3 μM) prepared in Krebs-Henseleit solution in the absence and presence of 300 μM GSH to check for possible quenching of dityrosine fluorescence by GSH.

The effect of GSH (3, 30 and 300 μM) and GSH mono-ethyl ester (3 μM) on coronary flow, myocardial function (heart rate, LVDP and RPP), and dityrosine concentration in the coronary effluent were recorded and compared with that of saline controls.

For the determination of myocardial cyclic 3',5'-guanosine monophosphate (cyclic GMP) and GSH levels, additional series of hearts were perfused according to

the protocol described above. The hearts were freeze-clamped with tongs cooled to the temperature of liquid nitrogen at 15, 17 or 25 min of aerobic perfusion prior to ischemia (0, 2 and 10 min after the administration of 300 μM GSH or vehicle, respectively), at the end of 20 min of global, no-flow ischemia, and after 1 or 10 min of reperfusion with the administration of 300 μM GSH or vehicle. The hearts were stored at -80 °C. Freeze-clamped hearts were pulverized under liquid nitrogen with a mortar and pestle.

Assay of myocardial cyclic GMP level

The powdered frozen heart tissue (200-250 mg) was suspended in 0.5 ml of a solution of ice-cold 100 mM Hepes: 5 mM EDTA: 2.2% perchloric acid and kept in ice for 15 min. The heart extracts were centrifuged (10,000 g, 2 min at 4 °C) and the supernatant was separated. 250 μl of the supernatant was neutralized with 40 μl of 1.1 M K_3PO_4 solution. This was centrifuged again (10,000 g, 2 min at 4 °C) and the supernatant was used for assay of cyclic GMP with an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor MI, USA). The pellet from the Hepes:EDTA:perchloric acid extract was kept for protein analysis. This was dissolved by boiling in 0.5 ml NaOH (2 M) for 10 min and then neutralized with 0.5 ml HCl (2 M). This was centrifuged (10,000 g, 2 min at 4 °C) and the supernatant was used for the determination of protein content using bicinchoninic acid reagent against bovine serum albumin as a standard. The level of cyclic GMP in myocardial tissue was expressed as pmol/mg protein.

Assay of myocardial GSH concentration

The powdered frozen heart tissue (50 mg) was suspended and homogenized in 1 ml of ice-cold 5% metaphosphoric acid. The heart extracts were centrifuged (3,000 g, 10 min at 4 °C) and the supernatant was separated. 80 µl of the supernatant was used for assay of GSH with a colorimetric assay kit (Calbiochem-Novabiochem Corporation, San Diego, CA, USA). The GSH concentration in the myocardial tissue was calculated as µmol/g tissue wet weight and is expressed as a percentage of the baseline level at 15 min of aerobic perfusion before the infusion of GSH or saline vehicle.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of the mean. For statistical analysis, one-way ANOVA or one-way repeated measures ANOVA were used for comparison of individual means between groups and within groups, respectively. Post-hoc testing with Fisher's least significant difference or Dunn's methods was used for parametric and non-parametric variables as appropriate. Student's *t* test was also performed to compare the difference of myocardial cyclic GMP levels between hearts treated with 300 μM GSH and controls. $P < 0.05$ was considered statistically significant.

RESULTS

1. Effects on coronary flow and mechanical function

Following 20 min global, no-flow ischemia, reperfusion resulted in a significant depression in the recovery of coronary flow and mechanical function (heart rate and LVDP) (Table 4.1) and RPP (Fig. 4.1) compared with values prior to the onset of ischemia. During aerobic perfusion, infusion of GSH at 300, but not 3 or 30 μM, significantly increased coronary flow (15.6 ± 0.3 vs. 13.9 ± 0.3 ml/min at 25 min and 15 min of aerobic perfusion, respectively, $P < 0.05$). The recovery of coronary flow was not different between the groups during reperfusion (Table 4.1). Glutathione caused a concentration-dependent improvement in the recovery of mechanical function during reperfusion compared with control hearts (Fig. 4.1, Table 4.1). The threshold for the response to GSH occurred at 3 μM whereas 30 and 300 μM GSH significantly enhanced the recovery of RPP during reperfusion. Increasing the concentration of GSH from 30 to 300 μM resulted in an earlier onset of recovery in mechanical function (Fig. 4.1). The enhanced recovery in RPP during reperfusion by GSH resulted from the improvement in both the recovery of heart rate and LVDP (Table 4.1).

2. Effects on $ONOO^-$ formation

The concentration of dityrosine in the coronary effluent, a marker of $ONOO^-$ formation, was increased in control hearts in the first 2 min of reperfusion (Fig. 4.2). The concentration of dityrosine in the coronary effluent peaked at 0.5 min of reperfusion and then gradually returned to near-baseline concentrations by 5 min of

reperfusion. There were no further changes in dityrosine concentrations over the remaining reperfusion period (data not shown). While 300 μM GSH had no effect on the fluorescent intensity of authentic standard solutions of dityrosine (0.1-3 μM, n = 3) in Krebs-Henseleit solution (data not shown), dityrosine concentrations in the coronary effluent were significantly reduced during aerobic perfusion in hearts treated with 30 and 300 μM GSH (Fig. 4.2). At 0.5 min of reperfusion, dityrosine concentrations were significantly reduced in hearts treated with 30 and 300, but not 3 μM, GSH. No significant differences in dityrosine concentrations were found between the groups at other time points during reperfusion.

3. Effects on myocardial cyclic GMP levels

In separate series of hearts, the effects of 300 μM GSH on myocardial cyclic GMP levels was evaluated. During aerobic perfusion, a 2.2-fold rise in myocardial cyclic GMP level occurred within 2 min of the infusion of 300 μM GSH during aerobic perfusion and then declined to modestly elevated levels after 10 min infusion (Fig. 4.3). Over the 20 min period of global, no-flow ischemia myocardial levels of cyclic GMP declined, an effect which was attenuated by GSH (64 % and 85 % of levels at 25 min of aerobic perfusion for control and GSH-treated hearts, respectively, $P < 0.05$, Fig. 4.3). Within the first min of reperfusion, the cyclic GMP level was then elevated to aerobic baseline values in control hearts, whereas there was a near 2-fold increase from baseline in GSH-treated hearts. During reperfusion, the elevated cyclic GMP level in GSH-treated hearts gradually fell and was no longer statistically different from controls at 10 min of reperfusion.

4. Effects on myocardial GSH concentrations

The baseline concentration of myocardial GSH at 15 min aerobic perfusion prior to the addition of GSH was 1.8 ± 0.1 μmol/g wet weight. Following the infusion of 300 μM GSH for 10 min, myocardial GSH concentrations were 2.0 ± 0.1 μmol/g wet weight ($P > 0.05$), and were also not significantly altered compared with 1.7 ± 0.1 μmol/g wet weight measured in control hearts after 25 min of aerobic perfusion (n = 3-4 in each group). At the end of 20 min global, no-flow ischemia, myocardial GSH concentrations modestly declined to 1.8 ± 0.1 and 1.4 ± 0.2 μmol/g wet weight for control and GSH-treated hearts, respectively, and were not significantly different between groups. With the infusion of 300 μM GSH during reperfusion, no further changes in myocardial GSH concentrations measured at 10 min reperfusion were observed (data not shown).

5. Effects of 3 μM GSH mono-ethyl ester

Mechanical function of hearts treated with 3 μM GSH mono-ethyl ester was significantly improved compared with control hearts and was equivalent to the protective action of 30 μM GSH (Fig. 4.4). Glutathione mono-ethyl ester caused significant increases in LVDP at 10 and 30 min of reperfusion (51 ± 9* and 52 ± 10* mmHg, n = 11, * $P < 0.05$ vs. controls) while there were no differences in heart rate during reperfusion (data not shown). There was a significant reduction in dityrosine concentration in the coronary effluent at 0.5 min of reperfusion (114 ± 11 % of 15 min aerobic baseline value, n = 11, $P < 0.05$ vs. control). Glutathione mono-ethyl ester also caused a significant vasodilator effect during aerobic perfusion (at 25 min:

14.5 ± 1.9 vs. 13.1 ± 1.9 ml/min at 15 min of aerobic perfusion, $P < 0.05$), whereas the coronary flow was not different from controls throughout the 30 min reperfusion period.

DISCUSSION

In this study, I showed that GSH could concentration-dependently improve the recovery of myocardial mechanical function in isolated rat hearts subjected to ischemia and reperfusion. I found that its protective actions are associated with the reduced release of endogenous $ONOO^-$ at reperfusion and with the stimulation of myocardial guanylate cyclase. This was shown by the decreased concentrations of dityrosine in the coronary effluent and the elevated level of cyclic GMP in the myocardial tissue, respectively, and was independent of changes in the tissue concentrations of GSH.

Nitric oxide and $O_2^{\bullet-}$ react at a diffusion-limited rate to form $ONOO^-$.[18] Yasmin et al previously demonstrated using the same ischemia-reperfusion model in hearts that the endogenous production of $ONOO^-$ is enhanced during early reperfusion[9] when there is a simultaneously increased production of NO as well as $O_2^{\bullet-}$.[19] Pharmacological measures to reduce the endogenous production of $ONOO^-$ during reperfusion such as the use of a NO synthase inhibitor, N^G-monomethyl-L-arginine, or a membrane permeable mimetic of superoxide dismutase, Mn (III)tetrakis(4-benzoic acid) porphyrin, are associated with an improved recovery of mechanical function during reperfusion.[9,20] In this study, I demonstrated an alternative method to reduce myocardial stunning during reperfusion. Glutathione may act as a unique scavenger of $ONOO^-$ as it is known to react with $ONOO^-$ to generate *S*-nitrosoglutathione.[8,11,21] *S*-nitrosoglutathione and other NO donors[9,22] have been shown to have a protective effect in myocardial ischemia-reperfusion or cardioplegic ischemic arrest.[23] In accordance with this, we showed that micromolar

concentrations of GSH protect from $ONOO^-$-induced inhibition of myocardial aconitase activity, a model enzyme system to study detrimental actions of $ONOO^-$,[14] and prevent the formation of dityrosine from the reaction of tyrosine with $ONOO^-$ in Krebs-Henseleit solution (Ferdinandy P. and Schulz R., unpublished data).

The pronounced increase in myocardial cyclic GMP levels within the first min of reperfusion, at the time when $ONOO^-$ production is maximal, indicates the acute stimulation of guanylate cyclase during early reperfusion. Mayer et al showed that the ability of $ONOO^-$ to stimulate guanylate cyclase is entirely dependent upon the presence of thiols and demonstrated the formation of *S*-nitrosoglutathione from GSH and $ONOO^-$ in a cell-free system.[8] I attempted to measure *S*-nitrosoglutathione in coronary effluent samples using high performance liquid chromatography.[8] Although I could measure *S*-nitrosoglutathione from the reaction of $ONOO^-$ (1 mM) and GSH (1 mM), the level of *S*-nitrosoglutathione in coronary effluent samples taken during aerobic perfusion or reperfusion was below the detection limit.

In crystalloid perfused isolated hearts, NO or NO donors may have protective effects in myocardial ischemia-reperfusion injury via a variety of possible mechanisms which include terminating $ONOO^-$-mediated lipid radical chain propagation[12] and stimulating the production of cyclic GMP which antagonizes the elevated levels of cytosolic Ca^{2+} observed during ischemia-reperfusion.[24,25] The improved recovery of mechanical function following cardioplegic ischemic arrest in *S*-nitrosoglutathione-treated hearts was associated with higher cyclic GMP levels.[23]

Lefer et al have reported the cardioprotective actions of exogenously supplied, low concentrations of $ONOO^-$ (0.1-1 μM) in ischemia-reperfusion which may be due

to inhibition of leukocyte-endothelial cell adhesion, preservation of coronary endothelium and a reduction in myocardial infarct size.[26,27] Indeed, a possible physiological role of $ONOO^-$ has been supported by studies showing that $ONOO^-$ can relax vascular smooth muscle,[28] inhibit platelet aggregation[11] and stimulate soluble guanylate cyclase,[8] each of these beneficial actions of $ONOO^-$ dependent upon its ability to react with thiols to form nitrosothiols.[8,11] I have found GSH caused coronary vasodilation in isolated rat hearts by NO- and soluble guanylate cyclase dependent mechanism,[7] likely due to the reaction of GSH with a low basal production of $ONOO^-$ in the normal aerobically perfused rat heart. Interestingly, this basal production of $ONOO^-$, as shown by the dityrosine fluorescence in the coronary effluent, is reduced during aerobic perfusion with the infusion of GSH (Fig. 4.2). Therefore, GSH can reduce the cytotoxic effects of $ONOO^-$ as well as potentiate its cytoprotective action via the generation of *S*-nitrosoglutathione.

Glutathione is also an important endogenous anti-oxidant. Glutathione can be oxidized to glutathione disulfide by hydrogen peroxide generated from the dismutation of $O_2^{\bullet-}$.[2] Supplementation with GSH may facilitate this reaction and reduce the amount of $O_2^{\bullet-}$ for the formation of $ONOO^-$. Reduced amounts of $O_2^{\bullet-}$ can also prolong the biological half-life of NO which leads to increased stimulation of guanylate cyclase.[29]

Administration of GSH (0.5-5 mM) to the culture medium of endothelial cells has been used to enhance their antioxidant defense mechanism.[30] The use of blood which contains 25-35 μM GSH in the arterial plasma of rats[31] to perfuse isolated hearts and the addition of GSH (3 mM) into cardioplegic solutions have been

demonstrated to improve cardiac function in heart transplantation.[32,33] Using isolated rat hearts perfused with perfusate containing 1.75 mM Ca^{2+} and subjected to 20 min of recirculating aerobic perfusion, 30 min of global and no-flow ischemia followed by 30 min of reperfusion, Tani et al did not show any protective action of 2-10 mM GSH infused during aerobic perfusion and throughout reperfusion.[5] However, they noted a concentration-dependent depression of heart function during aerobic perfusion with GSH, modest depression at 5 mM GSH and significant depression at 20 mM. I previously observed decreased mechanical function with infusion of GSH at 1 mM or greater during aerobic perfusion.[7] This may explain the lack of protective action with millimolar concentrations of GSH but a concentration-dependent protection on the recovery of cardiac function using micromolar concentrations of GSH. In addition, the finding that GSH could protect the heart without changing its total concentration is consistent with other studies.[6,30] This may suggest an extracellular and/or plasma membrane localization of the biochemical events involved in the cytoprotective actions of GSH.

In summary, GSH can improve the recovery of myocardial mechanical function after ischemia-reperfusion, an effect that may be related to the scavenging of $ONOO^-$ by GSH and the formation of an intermediate, which stimulates soluble guanylate cyclase.

Table 4.1 Effects of glutathione (GSH) on coronary flow, heart rate and left ventricular developed pressure (LVDP) in isolated perfused rat hearts subjected to 25 min aerobic perfusion, 20 min global, no-flow ischemia and 30 min reperfusion.

Hearts were treated either with saline vehicle (control) or GSH (3, 30 or 300 μM) for 10 min before ischemia and throughout the 30-min reperfusion period.

	coronary flow (ml/min)	heart rate (min^{-1})	LVDP (mmHg)
CONTROL (n = 22)			
15 min aerobic perfusion	14.0 ± 0.3	296 ± 7	81 ± 2
25 min aerobic perfusion	14.1 ± 0.2	305 ± 7	79 ± 2
10 min reperfusion	9.3 ± 0.6#	139 ± 47#	18 ± 6#
30 min reperfusion	8.1 ± 0.4#	187 ± 28#	24 ± 5#
3 μM GSH (n = 7)			
15 min aerobic perfusion	14.0 ± 0.3	276 ± 13	86 ± 2
25 min aerobic perfusion	14.0 ± 0.2	285 ± 13	82 ± 2
10 min reperfusion	9.5 ± 0.6#	184 ± 37#	33 ± 10#
30 min reperfusion	9.0 ± 0.5#	166 ± 44#	41 ± 12#
30 μM GSH (n = 12)			
15 min aerobic perfusion	13.7 ± 0.3	305 ± 11	78 ± 3
25 min aerobic perfusion	14.1 ± 0.4	308 ± 12	77 ± 3
10 min reperfusion	10.1 ± 0.8#	254 ± 42	34 ± 7#
30 min reperfusion	9.0 ± 0.8#	263 ± 19	47 ± 7#,*
300 μM GSH (n = 7)			
15 min aerobic perfusion	13.8 ± 0.3	324 ± 18	73 ± 4
25 min aerobic perfusion	15.6 ± 0.3#*	326 ± 16	73 ± 5
10 min reperfusion	9.7 ± 1.4#	291 ± 42*	57 ± 12*
30 min reperfusion	9.9 ± 1.6#	331 ± 50*	44 ± 10

Values represent mean ± s.e.m., # $P < 0.05$ vs. respective baseline value at 15 min of aerobic perfusion (one-way repeated measures ANOVA), * $P < 0.05$ vs. respective control value (one-way ANOVA).

Fig. 4.1 Glutathione (GSH) concentration-dependently increases the recovery of rate-pressure product (heart rate x left ventricular developed pressure) of isolated perfused rat hearts after 20 min of global, no-flow ischemia and 30 min reperfusion.

Hearts were treated with saline vehicle (control, ○, n = 22) or 3 (▲, n = 8), 30 (■, n = 12) or 300 (●, n = 7) μM GSH 10 min prior to ischemia and throughout the 30-min reperfusion period as indicated by the bars.

* $P < 0.05$ vs. control (one-way ANOVA)

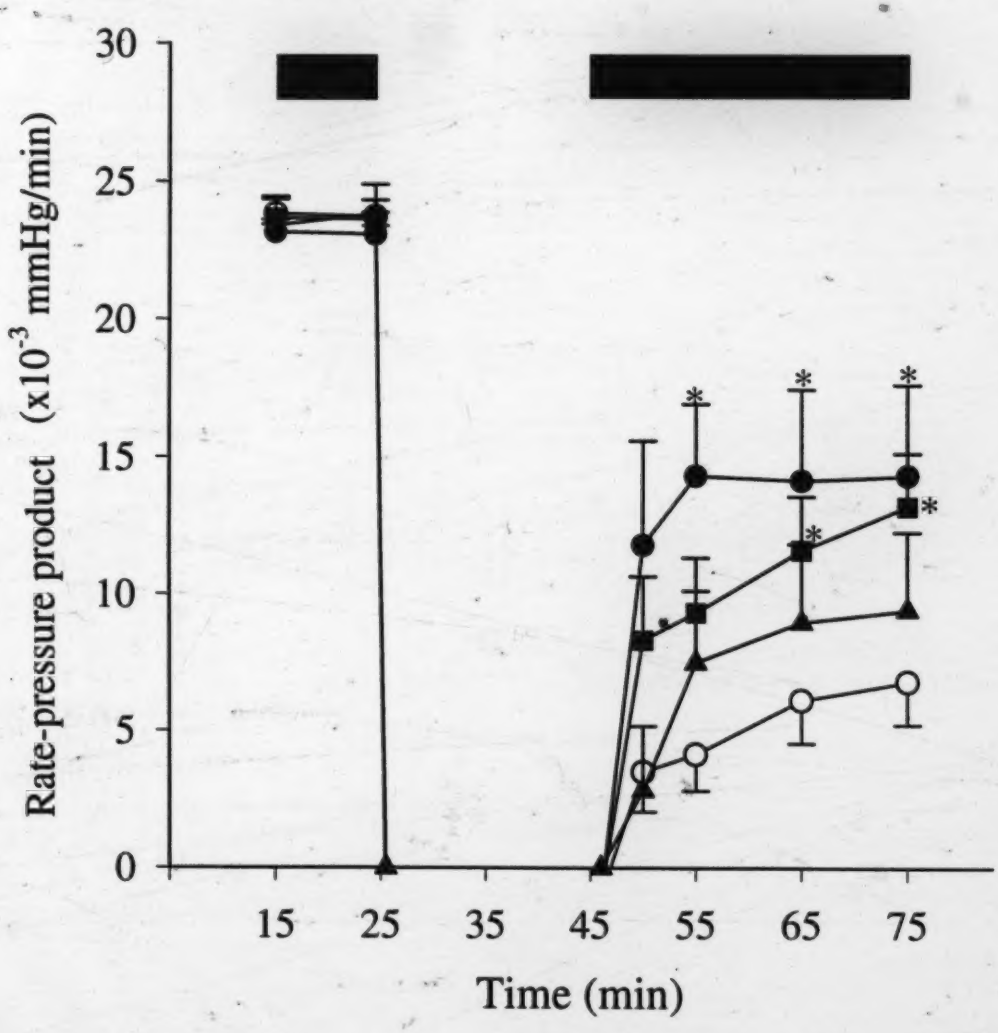


Fig. 4.2 Glutathione (GSH) reduces the dityrosine concentration in the coronary effluent from isolated perfused rat hearts subjected to 25 min aerobic perfusion, 20 min of global, no-flow ischemia and 30 min reperfusion.

Hearts were treated with saline vehicle (control, ○, n = 22) or 3 (▲, n = 8), 30 (■, n = 12) or 300 (●, n = 7) μM GSH 10 min prior to ischemia and throughout the 30-min reperfusion period. Values are expressed as a percentage of baseline dityrosine fluorescence of coronary effluent measured at 15 min of aerobic perfusion.

* $P < 0.05$ vs. control (one-way ANOVA)

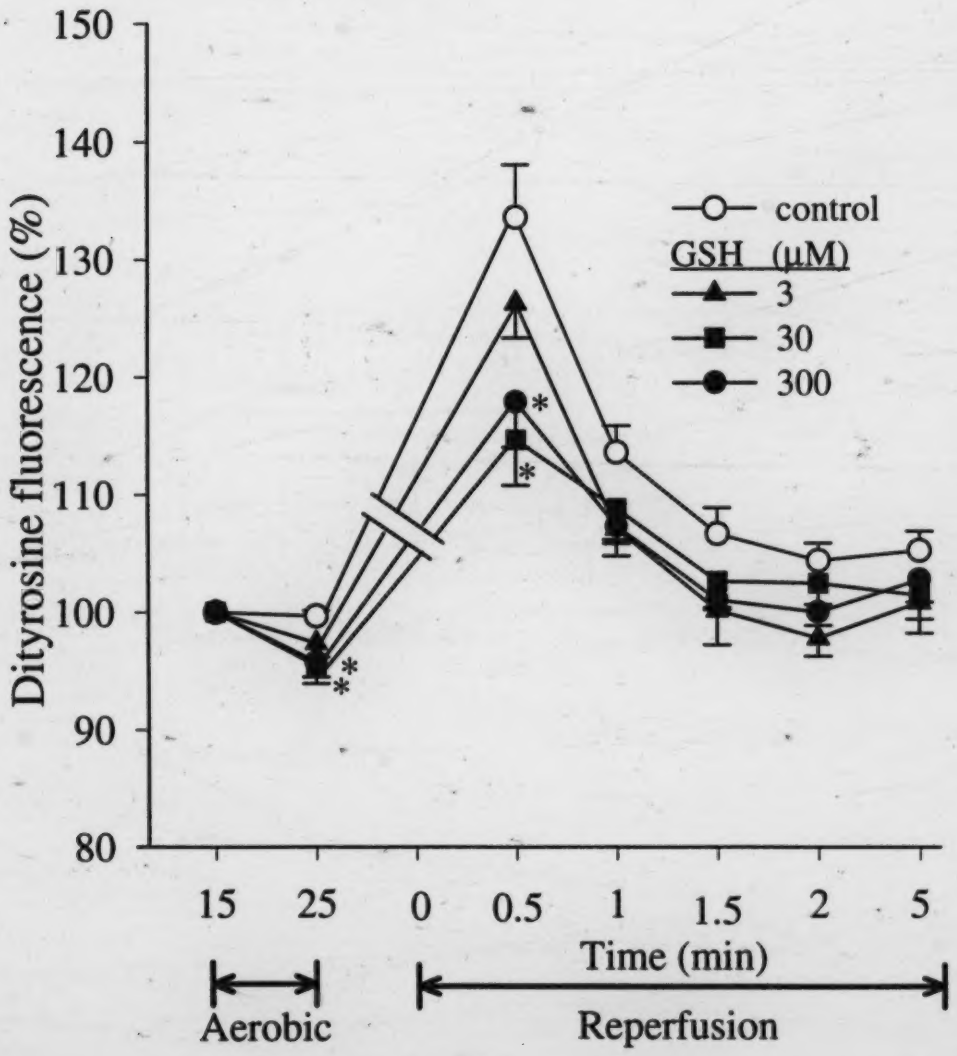


Fig. 4.3 Glutathione (GSH) increases myocardial cyclic GMP levels in isolated perfused rat hearts subjected to 25 min aerobic perfusion, 20 min of global, no-flow ischemia and 30 min reperfusion.

Hearts were treated with either saline vehicle (control, ○) or 300 μM GSH (●) 10 min prior to ischemia and throughout the 30-min reperfusion period.

* $P < 0.05$ vs. control (n = 4-9 in each group, Student's *t* test)

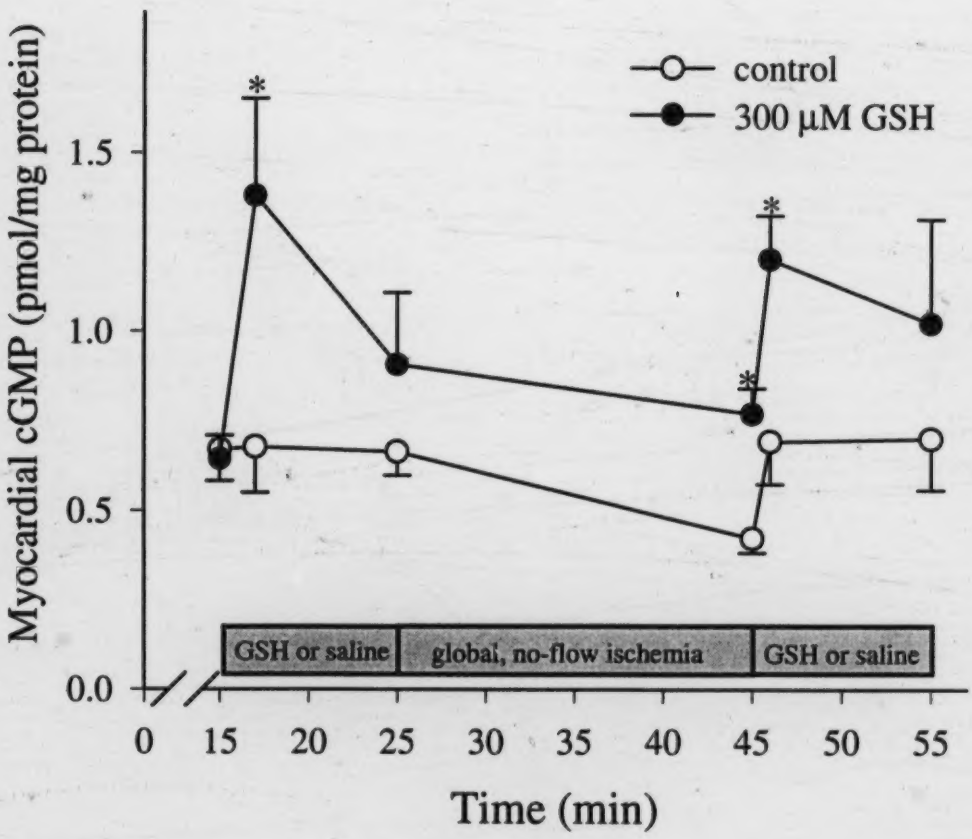
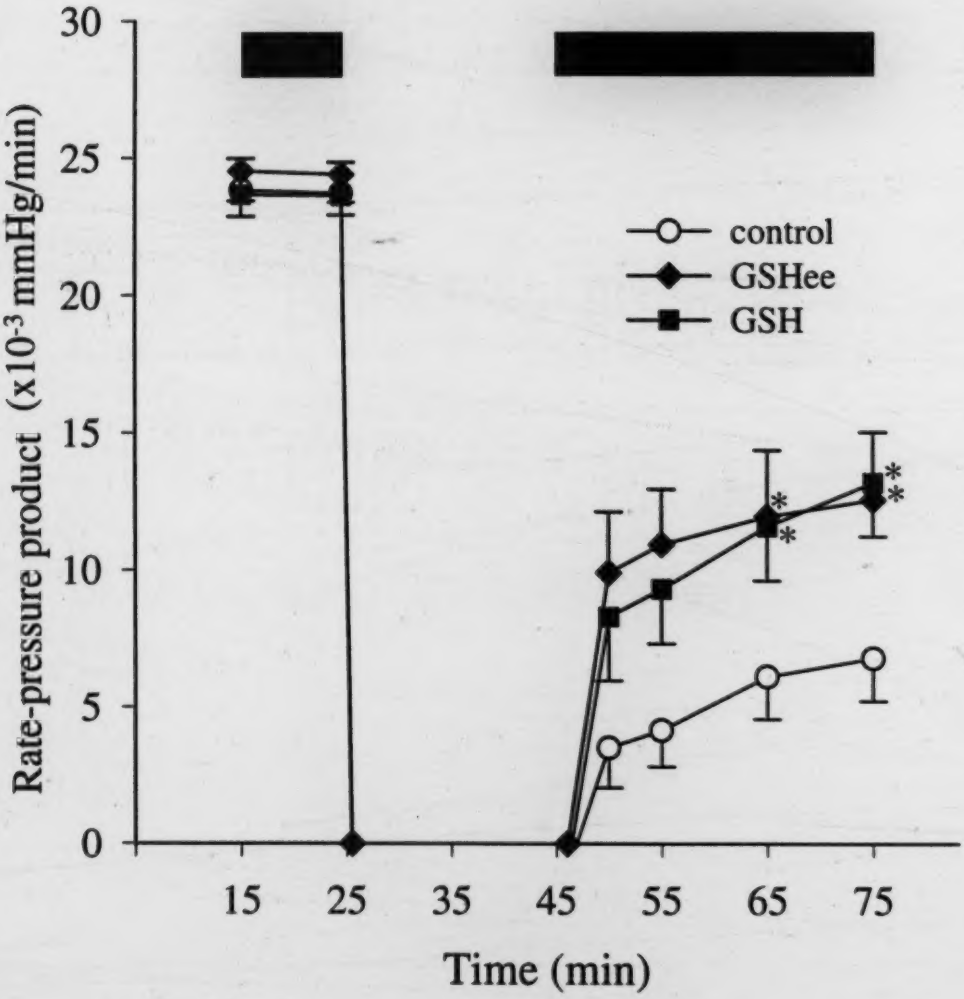


Fig. 4.4 Glutathione mono-ethyl ester (GSHee 3 μM) and glutathione (GSH 30 μM) increase the recovery of rate-pressure product (heart rate x left ventricular developed pressure) of isolated perfused rat hearts after 20 min of global, no-flow ischemia and 30 min reperfusion.

Hearts were treated with saline vehicle (control, ○, n = 22), 3 (▲, n = 11) μM GSHee or 30 (■, n = 12) μM GSH 10 min prior to ischemia and throughout the 30-min reperfusion period as indicated by the bars.

* $P < 0.05$ vs. controls (one-way ANOVA)



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CHAPTER V

INHIBITION OF MATRIX METALLOPROTEINASE – 2 IMPROVES RECOVERY OF MECHANICAL FUNCTION FOLLOWING ISCHEMIA-REPERFUSION IN ISOLATED RAT HEARTS

ABSTRACT

While matrix metalloproteinases (MMPs) contribute to collagen degradation following myocardial infarction, their role in acute changes in coronary flow and myocardial function during ischemia-reperfusion are unknown. Using spontaneously beating rat hearts perfused in Langendorff mode at constant pressure, we measured the release of gelatinolytic MMPs into the coronary effluent during aerobic perfusion and after ischemia-reperfusion. During aerobic perfusion, both pro MMP-2 (72 kDa), being the major gelatinase activity, and MMP-2 (64 kDa) were identified in coronary effluent by zymography. MMP-2 was also detected in the heart tissue by Western blot analysis. Using zymography, we found that inhibitors of metalloproteinases, *o*-phenanthroline, but not phosphoramidon (10 μM each), inhibited MMP-2 activity. *o*-Phenanthroline (3-100 μM), but not phosphoramidon (10 μM) or polyclonal antibody against MMP-2 (1.5-15 μg/ml), caused concentration-dependent vasodilation and decreased cardiac mechanical function during aerobic perfusion. Following 20 min of global, no-flow ischemia marked increases in the specific activities of pro MMP-2 (3.5-fold) and MMP-2 (20-fold) were found in the coronary effluent during the first 10 min of reperfusion. *o*-Phenanthroline (100 μM) or MMP-2 antibody (15 μg/ml), but not phosphoramidon (10 μM), improved the recovery of mechanical function during reperfusion. Pro MMP-2 and MMP-2 are continuously released into the coronary circulation of aerobically perfused hearts and their release is acutely elevated following ischemia-reperfusion. Inhibition of MMP-2 activity may be a novel therapeutic strategy to reduce myocardial ischemia-reperfusion injury.

INTRODUCTION

Matrix metalloproteinases (MMPs) belong to a family of zinc neutral endopeptidases.[1] The MMPs are synthesized in a latent form (pro-enzyme, pro MMP) and require activation by proteolytic cleavage of an amino-terminal domain,[2] or conformational changes induced by oxidative stress.[3] They are involved in the remodeling of the extracellular matrix in tissue during various physiological and pathological conditions such as embryonic development, inflammation and cancer.[4] Following myocardial infarction, increased activities of collagenase and other neutral proteinases in the heart have been shown.[5] This may be responsible for the rapid degradation of extracellular matrix collagen in myocardial infarction.[6] Recently, enhanced expression and increased activity of myocardial MMPs have been shown to contribute to ventricular remodeling in pigs with pacing-induced heart failure.[7]

Among the metalloproteinases, matrix metalloproteinase-2 (constitutive, pro-enzyme: pro MMP-2, pro-gelatinase A, 72-kDa; active enzyme: MMP-2, gelatinase A, 64 kDa) and matrix metalloproteinase-9 (inducible, pro-enzyme: pro MMP-9, pro-gelatinase B, 92-kDa; active enzyme: MMP-9, gelatinase B, 84 kDa) are involved in the degradation of collagen and intact collagen type IV, a major component of the basement membrane. While MMP-2 has been shown to be important in ventricular remodeling,[7] it was recently found in the releasate of human platelets during collagen-induced aggregation and it was shown that this enzyme mediated a novel pathway of platelet aggregation.[8] Substances which promote platelet aggregation often act as vasoconstrictors whereas inhibitors of platelet aggregation are vasodilators such as thromboxane and nitric oxide, respectively.[9,10] There is little

information on the expression or the acute release of MMP-2 from the heart under normal conditions. Furthermore, during reperfusion of the ischemic heart, the role of MMP-2 with regard to the re-establishment of normal coronary flow and mechanical contractile function has not been determined.

Using an isolated rat heart model, we investigated the release of MMP-2 into the coronary circulation and the role of MMP-2 in the regulation of coronary vascular tone and cardiac mechanical function during aerobic perfusion and following ischemia-reperfusion. We hypothesized that MMP-2 was released from the heart during aerobic perfusion and its release would be increased following ischemia-reperfusion. Furthermore we tested the hypothesis that inhibition of MMP-2 activity could improve the recovery of mechanical function following ischemia and reperfusion.

MATERIALS AND METHODS

This investigation conforms with the Guide to the care and use of experimental animals published by the Canadian Council on Animal Care (revised 1993).

Materials

Polyclonal anti-peptide antibodies were generated in rabbits using synthetic peptide corresponding to a fragment (N-G-K-E-Y-N-S-[ABA]-T) of the gelatin-binding domain of human MMP-2.[11] Rabbit IgG was purified according to Harlow & Lane[12] and was used as a negative control for the antibody against MMP-2. The supernatant from HT 1080 cells (American Type Culture Collection, Rockville, MD), which contains large amounts of MMP-2, was used as a standard. HT 1080 cells were maintained at 37 °C in a humidified chamber containing 5 % CO_2 in air in Eagle's MEM media with 10 % fetal calf serum at a concentration of 5 x 10^5 cells/ml. Before cell-conditioned medium was collected, the cells were washed 3 times with serum-free media and incubated at 37 °C for 24 hr with the phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (TPA) at 0.1 μM. The cell-conditioned media was separated from cells by centrifugation (1500 g for 10 min at room temperature), and the supernatant was aliquoted and stored at -80 °C. All other reagents were purchased from Sigma. *o*-Phenanthroline was dissolved in DMSO such that the final concentration reaching the heart never exceeded 0.2 % (v:v). For the other reagents, they were dissolved in deionized water.

Heart preparation and perfusion

Male Sprague-Dawley rats (250-300 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). Hearts were rapidly excised and briefly rinsed by immersion into an ice-cold Krebs-Henseleit solution. They were perfused via the aorta at a constant pressure of 60 mmHg with Krebs-Henseleit solution at 37 °C. The composition of Krebs-Henseleit solution was (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), and EDTA (0.5), and it was continuously gassed with 95 % O_2 / 5 % CO_2 (pH 7.4). The heart was surrounded by a water-jacketed glass chamber that maintained its temperature at 37 °C.

Spontaneously beating hearts were used in these experiments. A small latex balloon (size 4, Radnoti Glass Technology, Monrovia, CA, USA) connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve. The balloon was filled with deionized water (0.3-0.5 ml) to achieve a left ventricular end diastolic pressure of 8-12 mmHg. The transducer was connected to a Grass VI polygraph on which heart rate and left ventricular pressure were monitored. Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and end diastolic pressures of the left ventricle. The rate-pressure product (RPP) was calculated as the product of heart rate and LVDP, and was used as a measurement of cardiac mechanical function. Coronary flow was measured by an in-line ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) positioned proximal to the perfusion cannula.

The intervals between thoracotomy and attachment of the heart to the

perfusion system and between thoracotomy and beginning of stabilization period were less than 1 and 5 min, respectively. Using this perfusion protocol, hearts maintained a steady state of coronary flow, heart rate and LVDP for at least 80 min after stabilization as previously reported.[13]

Measurement of gelatinolytic MMPs in coronary effluent

The release of gelatinolytic MMPs into the coronary circulation was studied during aerobic perfusion and following ischemia-reperfusion. After 25 min of aerobic perfusion, 20 min of global and no-flow ischemia was induced by clamping the aortic inflow line and it was followed by 30 min of aerobic reperfusion by reopening the clamp. Samples of coronary effluent (6 ml) were collected for the determination of MMPs by zymography at 25 min of aerobic perfusion and at 1, 2, 5, 10, 20 and 30 min of reperfusion. As equal volume samples were collected, the duration time to collect the sample varied during reperfusion. The time required to collect the coronary effluent sample was approximately 0.43 min and between 0.43 and 1 min for the aerobic and reperfusion samples, respectively. The samples were stored at 4 °C and processed on the same day.

Samples of coronary effluent (6 ml) were concentrated 30-fold in volume using Centricon 10 concentrating vessels (Amicon Inc., Beverly, MA) (5000 g, 4 °C), and analyzed by zymography as previously described.[8] Briefly, non-heated sample concentrates were mixed with SDS sample buffer without 2-mercaptoethanol[14] and applied to 8 % polyacrylamide separating gels copolymerized with gelatin (2 mg/ml). After electrophoresis, the gels were washed three times at room temperature with 2 %

Triton X-100 to remove SDS and then incubated at 37 °C in 500 volumes of incubation buffer (50 mM Tris-HCl buffer at pH 7.5 containing with 0.15 M NaCl, 5 mM $CaCl_2$ and 0.05 % NaN_3) overnight to determine the activity of the enzymes. After incubation, the gels were stained with 0.05 % Coomassie Brilliant Blue G-250 in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and de-stained in aqueous 4 % methanol: 8 % acetic acid (v:v). Gelatinolytic activities were detected as transparent bands against the background of Coomassie Brilliant Blue-stained gelatin. Prestained molecular weight standards of the 47-205 kDa range, (Bio-Rad, Mississauga, ON) were used for reference. To quantitate the activities of the detected enzymes, zymograms were processed in the ScanJet 6100C scanner (Hewlett Packard, Boise, ID). The intensities of the separate bands were analyzed using SigmaGel measurement software (Jandel Corporation, San Rafael, CA).

In some experiments *o*-phenanthroline or phosphoramidon (10 μM each) were added to the incubation buffer. Incubation was carried out overnight followed by staining and analysis as described above. This was to investigate the inhibitory profile of these reagents on the gelatinolytic activities of MMP-2 and MMP-9.

Identification of MMP-2 in myocardial tissue by Western blot analysis

Aerobic control hearts were perfused for 2 min at 37 °C to rinse them free of blood and freeze-clamped for western blot analysis. Hearts were clamped with tongs cooled to liquid N_2 temperature and then rapidly frozen in liquid N_2. Freeze-clamped hearts were crushed by mortar and pestle under liquid N_2. Heart powder was then homogenized by sonication with a Vibra Cell™ apparatus (Sonics and Materials Inc.

Dunbery, CT) in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml soyabean trypsin inhibitor, 2 µg/ml aprotinin and 0.1% Triton X-100. The homogenate was centrifuged at 10,000 g at 4 °C for 10 min, and the supernatant was collected and stored at -80 °C until use.

Protein (40 µg) from heart extracts was applied to 7 % acrylamide gels. Protein concentration was determined by Bradford protein assay (BioRad, Mississauga, ON) using bovine serum albumin as a standard. Electrophoresis was carried out under reducing conditions according to Laemmli's method.[13] After electrophoresis, samples were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Schleicher and Schulell, Keene, NH) and probed with MMP-2 antibody (1 µg/ml). Using anti-rabbit IgG peroxidase conjugated antibody as the secondary antibody, the detection of protein bands was carried out with an ECL detection kit (Amersham, Oakville, ON).

Aerobic perfusion protocol

Following 20 min of stabilization, either *o*-phenanthroline or vehicle control (DMSO 0.2 %, v:v) were infused into aerobically perfused hearts via a side-port 15 cm proximal to the aortic cannula. They were infused at a constant rate of 0.1 ml/min by a syringe pump (Sage Instruments, Cambridge, MA, USA) and the concentration of the stock solutions was prepared based on an average coronary flow of 14 ml/min.

Five hearts were used for the study of concentration-dependent responses of *o*-phenanthroline (1-100 µM). The infusion at each concentration lasted for 10 min before the next higher concentration was tested. Coronary flow, heart rate and LVDP

were recorded and RPP was calculated at 1, 2, 5 and 10 min during the infusion at each concentration, by which time changes in functional parameters had either plateaued or reached near steady-state conditions. The recovery of hemodynamic parameters was recorded at 5, 10 and 15 min after stopping the infusion of the highest concentration.

Ischemia-reperfusion protocol

Following 15 min of stabilization, either *o*-phenanthroline (3-100 μM, an inhibitor of MMPs),[15] phosphoramidon (10 μM, an inhibitor of metalloproteinases but with weak inhibitory effect on MMPs),[14] or MMP-2 antibody (1.5-15 μg/ml) were infused for 10 min into the aerobically perfused heart at a constant rate of 0.1 ml/min. The concentration of the stock solutions was prepared based on an average coronary flow of 14 ml/min. Then 20 min of global, no-flow ischemia was induced and this was followed by 30 min of aerobic reperfusion. The test solutions were not infused during ischemia but were again infused for the first 10 min during reperfusion. DMSO (0.2 %, v:v) and rabbit IgG (15 μg/ml) were used as controls for *o*-phenanthroline and MMP-2 antibody, respectively.

STATISTICAL ANALYSIS

Data are expressed as mean ± standard error of mean. One-way ANOVA or ANOVA on ranks were used to compare the difference of continuous parametric or non-parametric variables between various treatment groups and controls, respectively. One-way repeated measures ANOVA was used for within-group comparison of variables at different time points of the experiment. Fisher's least significant difference and Dunn's test were used for post-hoc testing as appropriate. Student's *t* test was also used to compare the difference between two groups of parametric variables. $P < 0.05$ was considered statistically significant.

RESULTS

1. The release of pro MMP-2 and MMP-2 into coronary effluent in the isolated rat heart

At 25 min of aerobic perfusion, we found three bands of gelatinolytic activity (75, 72 and 64 kDa) in the coronary effluent (Fig. 5.1A). These activities were inhibited by 10 μM *o*-phenanthroline but not by phosphoramidon at the same concentration (Fig. 5.1B). The 72 kDa and 64 kDa forms were identified as pro MMP-2 and MMP-2 respectively by comparison to the standard and the inhibitory profile of *o*-phenanthroline and phosphoramidon. Quantitative analysis of the zymograms of the coronary effluents showed that pro MMP-2 is the major gelatinolytic activity, followed by 64 kDa MMP-2 and an unidentified 75 kDa activity (Fig. 5.1C). Pro MMP-9 (92 kDa) and MMP-9 (84 kDa) activities were not found in the coronary effluent.

Following 20 min global, no-flow ischemia, the temporal change of the specific activity of MMP-2 in coronary effluent during reperfusion is illustrated in Fig. 5.2. During early reperfusion, there was an acute release of pro MMP-2 into the coronary effluent from the heart as shown by the increase in 72 kDa gelatinolytic activity. The activity of pro MMP-2 in the coronary effluent peaked at the first min of reperfusion (3.5-fold of baseline values at 25 min aerobic perfusion) and gradually decreased to baseline aerobic values after 10 min of reperfusion. Also, an increase in the activity of 64 kDa form was found in the coronary effluent following reperfusion. This activity peaked at 5 min of reperfusion (20-fold of baseline values at 25 min aerobic perfusion) and had a lower maximum activity in the coronary effluent than

that of pro MMP-2 (Fig. 5.2). After 20 min reperfusion the MMP-2 activity was no longer different from baseline aerobic values. Gelatinolytic activity of the unidentified 75 kDa form was also increased during reperfusion.

2. The presence of MMP-2 in the isolated rat heart

MMP-2 was detected from aerobically perfused heart tissue by Western blot analysis (n = 3, Fig. 5.3).

3. The effect of o-phenanthroline in aerobically perfused hearts

Infusion of *o*-phenanthroline (1-100 μM) caused a cumulative concentration-dependent increase in coronary flow with a threshold concentration of 3 μM and the maximum change at 30 μM (from baseline of 13.2 ± 0.4 to 16.5 ± 0.5 ml/min; 25 % increase, n = 5, Fig. 5.4A). The increase in coronary flow occurred within the first min whereas maximum change was seen within 5 min following a change in *o*-phenanthroline concentration. The infusion of 100 μM *o*-phenanthroline caused a modest decrease in coronary flow compared with that was seen at 30 μM *o*-phenanthroline, but it was still significantly higher than baseline (15.4 ± 0.4 ml/min, 17 % above baseline). The increase in coronary flow by *o*-phenanthroline was not readily reversible, because coronary flow remained above baseline levels 15 min after the cessation of 100 μM *o*-phenanthroline infusion (15.2 ± 0.5 ml/min, Fig. 5.4A).

Compared with the vehicle control group, *o*-phenanthroline (1-100 μM) caused a significant concentration-dependent depression on heart rate, but not LVDP, resulting in a significant decrease in RPP (Fig. 5.4B-D). This significant depression

of heart rate and RPP occurred at 10 μM *o*-phenanthroline and became maximum at the highest concentration tested (100 μM, resulting in a 51 % and 53 % decrease from baseline RPP and heart rate, respectively). The significant decline in RPP and heart rate was completely recovered 15 min after stopping the infusion (Fig. 5.4B and D). Transient depression of LVDP occurred at the first 2 min following a change in *o*-phenanthroline concentration, but LVDP at 10 min of each concentration was not significantly different from baseline (Fig. 5.4C). Following the discontinuation of *o*-phenanthroline infusion, LVDP returned to baseline values.

4. The effect of o-phenanthroline and phosphoramidon in hearts subjected to ischemia-reperfusion

Following 20 min global, no-flow ischemia, coronary flow, heart rate, LVDP and RPP during reperfusion were significantly depressed than the respective baseline values at 25 min of aerobic perfusion immediately prior to the onset of ischemia (Table 5.1, Fig. 5.5). *o*-Phenanthroline caused a concentration-dependent improvement in the recovery of mechanical function during reperfusion compared with control hearts (Fig. 5.5, Table 5.1). This was accompanied by a concentration-dependent decrease in the RPP during aerobic perfusion (Fig. 5.5). The threshold for the improvement by *o*-phenanthroline occurred at 3 μM whereas 100 μM *o*-phenanthroline significantly enhanced the recovery of RPP during reperfusion. The enhanced recovery in RPP during reperfusion by *o*-phenanthroline resulted from the improvement in both the recoveries of heart rate and LVDP (Table 5.1).

During aerobic perfusion, infusion of *o*-phenanthroline at 100, but not at 3 or

10 μM, significantly increased the baseline coronary flow (Table 5.1). The coronary flow at 30 min of reperfusion was significantly elevated in the hearts treated with 100, but not 3 or 10 μM *o*-phenanthroline.

Phosphoramidon (10 μM) had no effect on the mechanical function and coronary flow in aerobically perfused heart (data not shown). In hearts treated with phosphoramidon, the mechanical function and coronary flow during reperfusion was not different from that of controls (at 30 min of reperfusion, RPP: 6.8 ± 4.1 vs. 7.6 ± 1.6 x 10^{-3} mmHg/min; coronary flow: 7.5 ± 2.1 vs. 9.0 ± 0.6 ml/min, respectively, $P > 0.05$, n = 4 in each group, Student's *t* test).

5. The effect of MMP-2 antibodies in hearts subjected to ischemia-reperfusion

Infusion of MMP-2 antibody at 1.5-15 μg/ml did not cause any significant change in the coronary flow and cardiac mechanical function during aerobic perfusion (Table 5.2, Fig. 5.6). There was a concentration-dependent improvement in the recovery of mechanical function in hearts treated with MMP-2 antibody compared with rabbit IgG (15 μg/ml) treated controls (RPP shown in Fig. 5.6). MMP-2 antibody caused a threshold improvement in the recovery of RPP at 5 μg/ml, and at 15 μg/ml caused improvements in RPP after 20 and 30 min of reperfusion compared with control hearts ($P = 0.08$, one-way ANOVA; $P < 0.05$, Student's *t* test). At 30 min of reperfusion, hearts treated with 15 μg/ml MMP-2 antibody had higher LVDP than that of controls ($P = 0.10$, one-way ANOVA; $P < 0.05$, Student's *t* test), while no differences in coronary flow or heart rate between groups were observed (Table 5.2).

DISCUSSION

In this study, we have demonstrated for the first time that MMP-2 (gelatinase A, both the pro-enzyme and active form) is released into the coronary circulation in aerobically perfused rat hearts and that pro MMP-2 constitutes the major gelatinase activity in the coronary effluent. We did not identify any MMP-9 (gelatinase B) activity in the coronary effluent. MMP-2 was expressed in heart tissue, similar to the recent study by Spinale et al[7] which demonstrated the presence of MMP-2 in porcine heart extract. The expression of MMP-2 has been shown in endothelial cells, endocardial, sub-endocardial layers as well as in mesenchymal cells.[8,16,17] In addition, I also found an unidentified gelatinase at 75 kDa. While this 75 kDa form may be an additional form of MMP-2, it has been recently shown to be an activated form of MMP-9 by neutrophil elastase.[18] The significance of this form is not known.

There is an acute release of MMP-2 (both the pro-enzyme and active form) into the coronary effluent with increased specific activity during early reperfusion. In addition to its well-studied role in tissue remodeling, the findings in this study suggest a novel role for MMP-2 in the development of myocardial stunning following ischemia. This acute role of MMP-2 is supported by (1) the association between the release of MMP-2 during reperfusion and the recovery of cardiac mechanical function in hearts subjected to 15 to 25 min of ischemia, and (2) the inhibition of MMP-2 by *o*-phenanthroline and MMP-2 antibody improves the recovery of mechanical function following ischemia-reperfusion. Phosphoramidon, an inhibitor of metalloproteinases but with weak inhibitory effect on MMPs, did not, however, improve mechanical function during reperfusion. This supports the notion that MMP-2, rather than other

MMPs, is the enzyme associated with myocardial stunning following ischemia-reperfusion. In rats subjected to focal stroke by permanent middle cerebral artery occlusion, Romanic et al demonstrated the progressive expression of MMP-2 and MMP-9 over 6 hr to 15 days by Western blot and zymography analysis.[19] They, however, showed a 30 % reduction in infarct size with the intravenous administration of MMP-9 antibody compared with non-immune antibody controls. Although the acute role of MMPs may be tissue specific, the findings compliment that of Romanic's study and suggest that MMPs may also contribute to the acute injury in addition to its well-known effect on tissue remodeling.

The inhibition of MMP-2 with *o*-phenanthroline, but not MMP-2 antibody, caused a depressed mechanical function during aerobic perfusion. While this depressed cardiac function may contribute to the improved recovery of mechanical function following ischemia-reperfusion, this also suggests that both specific and non-specific effects of *o*-phenanthroline on other MMPs and zinc-dependent enzymes in addition to the inhibition of MMP-2, (rather that MMP-2 itself) plays an important role in the regulation of coronary flow and cardiac function during aerobic perfusion.

Several mechanisms may explain the association between MMP-2 and impaired contractile function during reperfusion. Firstly, it has been shown that a decrease in collagen content occurred as early as at 30 min of reperfusion following ischemia which was related to an increased activity of collagenase.[6] Hence, the release and increased activity of MMP-2 could be involved in the modification of the matrix and excitation-contraction coupling mechanism and the associated myocardial stunning. Secondly, the effect of MMP-2 in the control of ion pump activity[20]

particularly the intracellular Ca^{2+} concentration, may also be related to its effect on the recovery of mechanical function of heart following ischemia-reperfusion.[21] Inhibition of MMP-2 may alleviate the rise in intracellular Ca^{2+} concentration resulting in better recovery of cardiac function following ischemia-reperfusion.

Activation of MMPs involves the breakage of a zinc-cysteine bond and the opening of the active catalytic site of the enzyme.[22] While proteolysis constitutes an important pathway for the activation of MMPs, MMPs can also be activated via conformational changes in the presence of oxidants such as peroxynitrite ($ONOO^-$) and glutathione disulfide.[3,8] Indeed, $ONOO^-$ can activate procollagenase in activated neutrophils.[3] We detected MMP-2 activity in the coronary effluent during aerobic perfusion and a 20-fold increase of MMP-2 activity during reperfusion following ischemia. While it is possible that $ONOO^-$ might be produced in aerobically perfused hearts under basal conditions in a hyperoxic environment, an acute and simultaneous release of high levels of both nitric oxide and superoxide, forming $ONOO^-$, has been shown within sec to min of reperfusion of the ischemic heart.[23,24] The acute release of $ONOO^-$ at reperfusion following ischemia contributes to myocardial stunning. Thus the production of $ONOO^-$ in the heart may be responsible for the activation of pro MMP-2 during aerobic perfusion and ischemia-reperfusion. While the action of $ONOO^-$ in the release of MMP-2 is not known, it is possible that $ONOO^-$ activates MMP-2 that may contribute to decreased myocardial function during reperfusion. Moreover, Frears et al have demonstrated the inactivation of a tissue inhibitor of MMPs by $ONOO^-$ in vitro.[25] Thus, the adverse effect of MMP-2 could be unhindered with inactivated or insufficient local concentration of tissue inhibitor of MMPs due to

the increased production of $ONOO^-$ in ischemia-reperfusion.

In summary, MMP-2 is released into the coronary circulation of aerobically perfused hearts and the release is acutely elevated following ischemia-reperfusion, and we have shown that MMP-2 plays a detrimental role in the recovery of cardiac function following ischemia-reperfusion. Inhibition of MMP-2 may be a novel therapeutic strategy to reduce myocardial stunning injury.

Table 5.1 Effects of *o*-phenanthroline (PNT) on coronary flow, heart rate and left ventricular developed pressure (LVDP) in isolated perfused rat hearts subjected to 25 min aerobic perfusion, 20 min global, no-flow ischemia and 30 min reperfusion.

Hearts were treated either with PNT (3, 10 and 100 μM) or dimethyl sulfoxide (0.2% v:v, vehicle control) for 10 min before ischemia and first 10 min of reperfusion.

	Coronary flow (ml/min)	heart rate (min^{-1})	LVDP (mmHg)
CONTROL (n = 15)			
15 min aerobic perfusion	13.3 ± 0.3	278 ± 6	82 ± 2
25 min aerobic perfusion	13.7 ± 0.3	278 ± 6	83 ± 3
10 min reperfusion	10.2 ± 0.9#	233 ± 67	21 ± 8#
30 min reperfusion	8.5 ± 0.5#	216 ± 28#	46 ± 8#
3 μM PNT (n = 12)			
15 min aerobic perfusion	13.2 ± 0.2	282 ± 12	83 ± 2
25 min aerobic perfusion	13.3 ± 0.4	281 ± 9	72 ± 4
10 min reperfusion	13.2 ± 0.8*	310 ± 67	40 ± 10#
30 min reperfusion	11.4 ± 0.7#	420 ± 76#*	48 ± 10#
10 μM PNT (n = 9)			
15 min aerobic perfusion	12.4 ± 0.2	286 ± 7	78 ± 2
25 min aerobic perfusion	13.0 ± 0.2	302 ± 8	62 ± 2#
10 min reperfusion	10.4 ± 0.4#	278 ± 43	23 ± 5#
30 min reperfusion	9.4 ± 0.4#	253 ± 6	53 ± 4#
100 μM PNT (n = 6)			
15 min aerobic perfusion	12.8 ± 0.3	272 ± 11	87 ± 2
25 min aerobic perfusion	14.3 ± 0.6#	237 ± 13	71 ± 4#
10 min reperfusion	15.8 ± 0.3#*	222 ± 14#	61 ± 4#*
30 min reperfusion	13.2 ± 0.4*	272 ± 19*	84 ± 4*

Values represent mean ± s.e.m., # $P < 0.05$ vs. respective baseline value at 15 min of aerobic perfusion (one-way repeated measures ANOVA), * $P < 0.05$ vs. corresponding value from control group (one-way ANOVA).

Table 5.2 Effects of MMP-2 antibody (MMP-2 Ab) on coronary flow, heart rate and left ventricular developed pressure (LVDP) in isolated perfused rat hearts subjected to 25 min aerobic perfusion, 20 min global, no-flow ischemia and 30 min reperfusion.

Hearts were treated either with MMP-2 Ab (1.5, 5 and 15 µg/ml) or rabbit IgG (Control, 15 µg/ml) for 10 min before ischemia and first 10 min of reperfusion.

	Coronary flow (ml/min)	heart rate (min^{-1})	LVDP (mmHg)
CONTROL (n = 5)			
15 min aerobic perfusion	12.8 ± 0.6	292 ± 8	78 ± 2
25 min aerobic perfusion	12.8 ± 0.5	292 ± 10	78 ± 2
20 min reperfusion	8.6 ± 0.9#	306 ± 92	24 ± 8#
30 min reperfusion	8.6 ± 0.8#	298 ± 63	22 ± 8#
1.5 µg/ml MMP-2 Ab (n = 4)			
15 min aerobic perfusion	13.5 ± 0.6	260 ± 9	83 ± 3
25 min aerobic perfusion	13.3 ± 0.5	265 ± 7	81 ± 3
20 min reperfusion	8.5 ± 1.8#	285 ± 65	22 ± 36#
30 min reperfusion	8.5 ± 1.6#	292 ± 145	20 ± 11#
5 µg/ml MMP-2 Ab (n = 4)			
15 min aerobic perfusion	13.3 ± 0.5	320 ± 28	75 ± 6
25 min aerobic perfusion	13.5 ± 0.3	323 ± 26	73 ± 5
20 min reperfusion	7.8 ± 1.0#	233 ± 83	23 ± 11#
30 min reperfusion	7.8 ± 0.9#	233 ± 83	33 ± 12#
15 µg/ml MMP-2 Ab (n = 4)			
15 min aerobic perfusion	12.5 ± 0.3	285 ± 15	79 ± 3
25 min aerobic perfusion	11.8 ± 0.5	265 ± 5	86 ± 3
20 min reperfusion	8.0 ± 1.2#	260 ± 14	46 ± 3#
30 min reperfusion	8.0 ± 1.3#	280 ± 27	51 ± 5#*

Values represent mean ± s.e.m., # $P < 0.05$ vs. respective baseline value at 15 min of aerobic perfusion (one-way repeated measures ANOVA), * $P < 0.05$ vs. corresponding value from control group (Student's *t* test).

Fig. 5.1 Predominance of pro MMP-2 in coronary effluents from isolated rat hearts at 25 min of aerobic perfusion by zymography.

(A) A representative zymogram. Left lane: HT1080 cell derived standards showing MMP-2 (64 kDa), pro MMP-2 (72 kDa) and MMP-9 (92 kDa). Right lane: coronary effluent collected at 25 min of aerobic perfusion. There was gelatinase activity in the 64-75 kDa range with a predominance of activity at 72 kDa. No gelatinase activity was found in the 82-92 kDa range. (B) Gelatinase activities of the 25 min aerobic perfusion sample were confirmed by the complete inhibition of gelatinase activities in gels containing *o*-phenanthroline but not phosphoramidon (10 μM each). (C) Quantification of the relative amount of MMP-2 (64 kDa), pro MMP-2 (72 kDa) and 75 kDa gelatinase activity in coronary effluents collected at 25 min of aerobic perfusion by densitometric measurement of zymograms (n = 5).

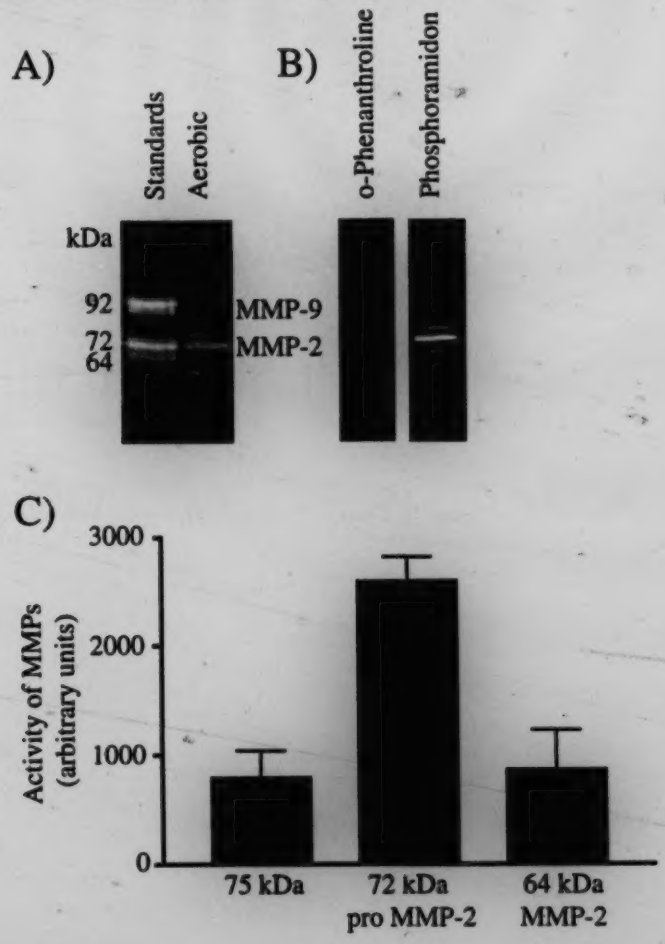


Fig. 5.2 The acute release of pro MMP-2 (72 kDa) and MMP-2 (64 kDa) into the coronary effluent of isolated rat hearts at 25 min of aerobic perfusion and during reperfusion after 20 min of global, no-flow ischemia.

Upper panel: a representative zymogram from one experiment.

Lower panel: quantification of relative intensities of pro MMP-2 and MMP-2 on zymograms in isolated rat hearts (n = 8).

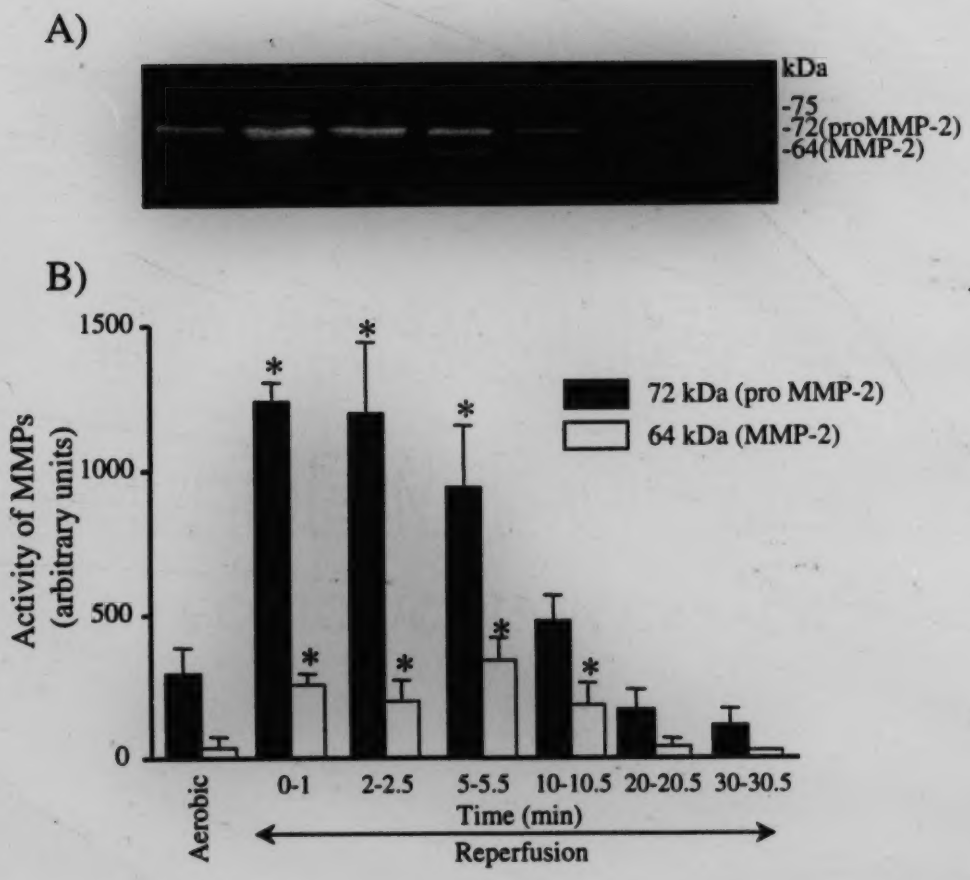


Fig. 5.3 Presence of MMP-2 in the heart extract by Western blot analysis

Left lane: HT1080 cells derived standards showing MMP-2 (64 kDa).

Right lane: Extract from a heart that was aerobically perfused for 2 min at 37 °C to rinse it free of blood, followed by freeze-clamping and preparation of heart extract.

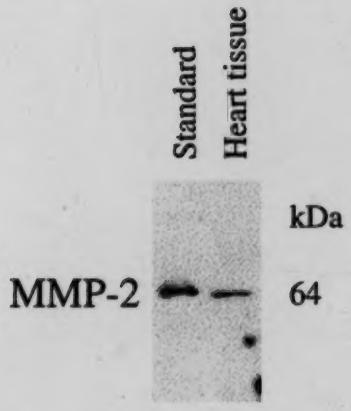


Fig. 5.4 Concentration-dependent actions of *o*-phenanthroline (PNT, n = 5) on coronary flow and mechanical function in isolated rat hearts

Increasing concentrations of PNT were infused at 10-min intervals and (A) coronary flow, (B) heart rate, (C) left ventricular developed pressure (LVDP) and (D) rate-pressure product (heart rate x LVDP) were recorded at 1, 2, 5 and 10 min of infusion at each concentration. A 15 min recovery period after infusion of 100 μM is also shown. Numbers in bar indicate concentration (μM). Vehicle control (hearts treated with dimethyl sulfoxide 0.2% v:v) is indicated by the open circles.

* $P < 0.05$ vs. 20 min baseline value (one-way repeated measures ANOVA)

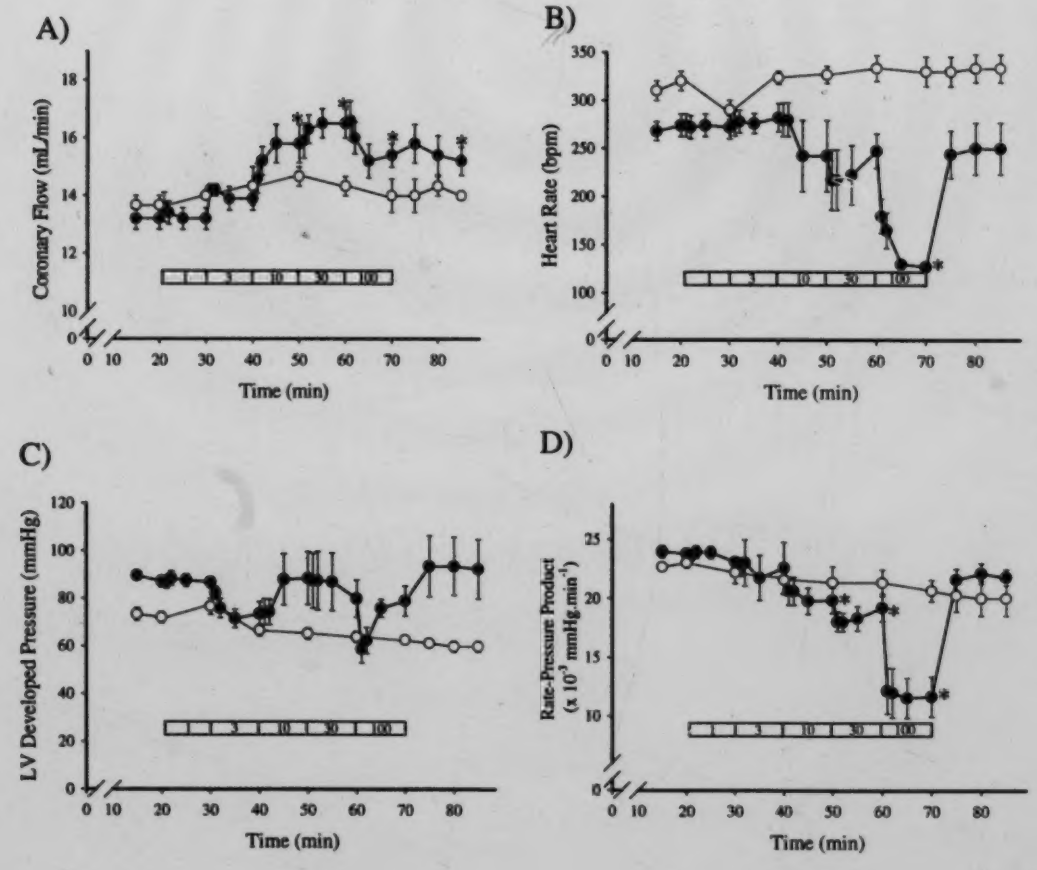


Fig. 5.5 ***o*-Phenanthroline (PNT) increases the recovery of rate-pressure product (heart rate x left ventricular developed pressure) in isolated rat hearts after 20 min of global, no-flow ischemia and 30 min reperfusion.**

Hearts were treated with dimethyl sulfoxide (0.2% v:v, vehicle control, ○, n = 15) or 3 (▲, n = 12), 10 (■, n = 9) or 100 (●, n = 6) μM PNT 10 min prior to ischemia and first 10 min of the 30-min reperfusion period as indicated by the bars.

* $P < 0.05$ vs. control (one-way ANOVA)

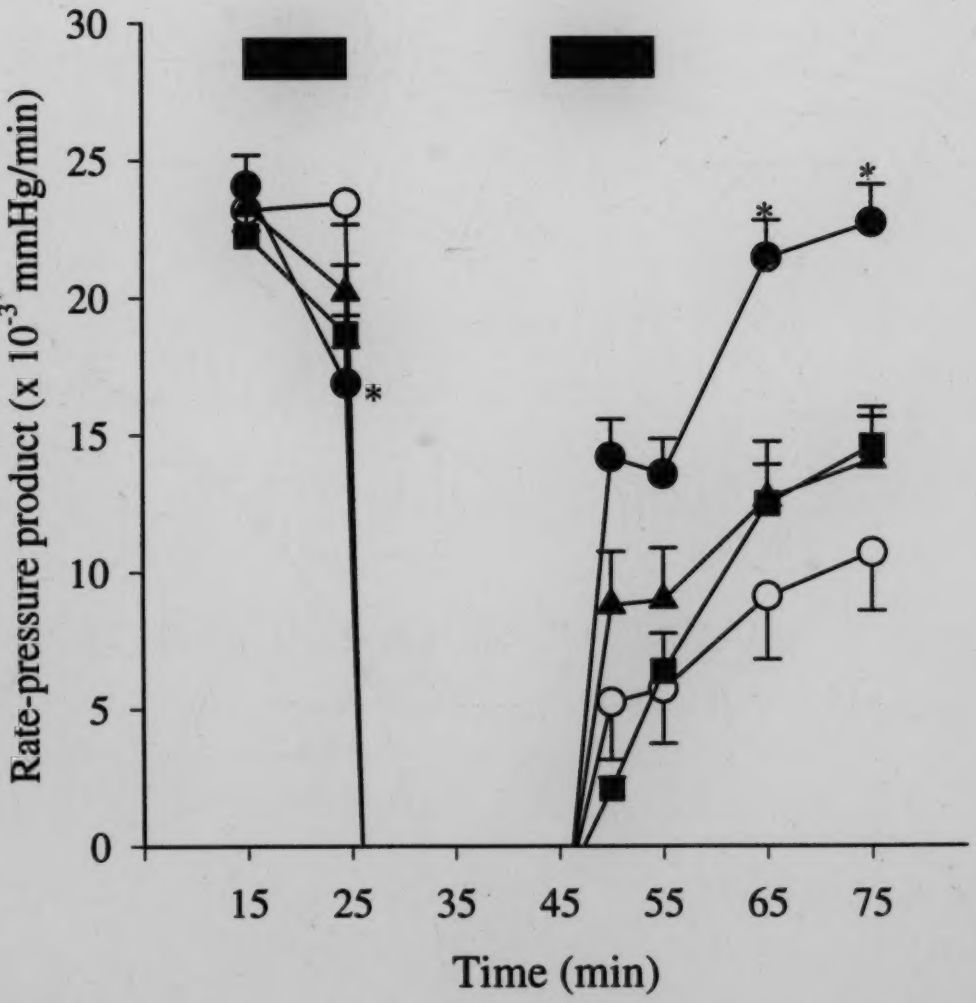
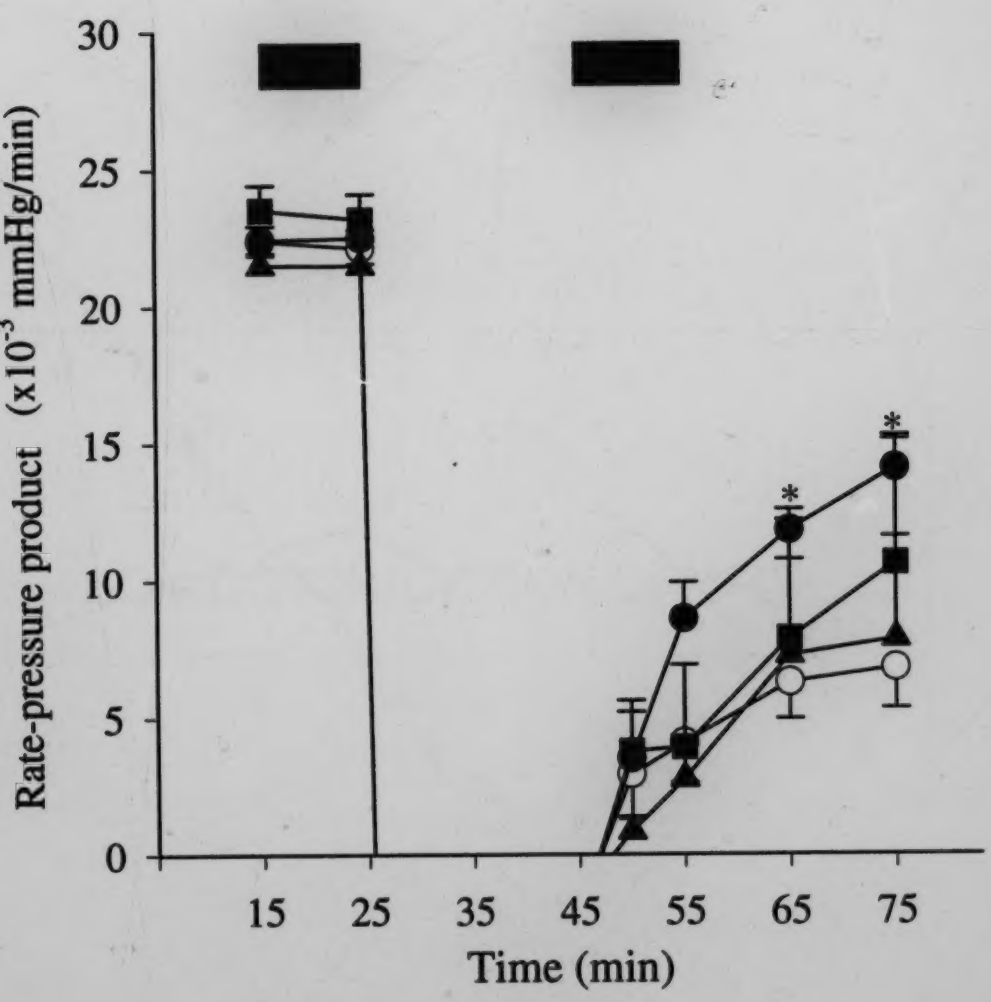


Fig. 5.6 MMP-2 antibody (MMP-2 Ab, 1.5-15 μg/ml) concentration-dependently increases rate-pressure product (heart rate x left ventricular developed pressure) in isolated rat hearts after 20 min of global, no-flow ischemia and 30 min reperfusion.

Hearts were treated with either rabbit IgG (vehicle control, ○, 15 μg/ml, n = 5) or 1.5 (▲, n = 4), 5 (■, n = 4) or 15 (●, n = 4) μg/ml MMP-2 Ab 10 min prior to ischemia and first 10 min of the 30-min reperfusion period as indicated by the bars.

* $P < 0.05$ vs. control (Student's *t* test)



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CHAPTER VI

PLATELET ACTIVATION WITH ELEVATED PLASMA MATRIX METALLOPROTEINASE-2 LEVELS DURING EXTRACORPOREAL MEMBRANE OXYGENATION IN NEONATES

A version of this chapter has been submitted for publication. Cheung P-Y, Sawicki G, Salas E, Etches PC, Schulz R, Radomski MW. (1998) Lancet.

ABSTRACT

Extracorporeal membrane oxygenation (ECMO) is based on the use of prolonged extracorporeal circulation for temporary respiratory or cardiac support. Bleeding associated with thrombocytopenia and abnormal platelet aggregation is the most frequent complication of ECMO, however, the mechanism of platelet dysfunction remains unknown. I investigated the role of matrix metalloproteinase-2 (MMP-2) that has recently been shown to mediate a novel pathway of platelet aggregation, in the platelet dysfunction induced by ECMO. In a prospective study of 10 neonates treated with ECMO from June 1996-March 1997, I found that the procedure caused a time-dependent platelet activation as evidenced by thrombocytopenia, decreased collagen (10 μg/ml)-induced platelet aggregation in whole blood and increased release of the soluble P-selectin from platelets. Plasma MMP-2 activity was elevated during ECMO, whereas the tissue inhibitor of MMP-2 (TIMP-2) remained below detectable limits. Plasma soluble P-selectin levels significantly correlated with plasma MMP-2 ($r = 0.61$, $P < 0.001$), but not with MMP-9 activities. Increased lipid hydroperoxides, a marker of oxidative stress, were also found in plasma. These changes of platelet function were not associated with endothelial activation as shown by normal plasma levels of soluble E-selectin and nitric oxide metabolites, nitrite and nitrate. Platelet dysfunction persisted despite repeated platelet transfusions to maintain platelet counts > 100×10^9/L. The findings suggest that MMP-2 may play a role in the development of platelet dysfunction brought about by extracorporeal circulation procedures, such as ECMO. Interventions to reduce platelet activation may improve the haemostatic-thrombotic imbalance.

INTRODUCTION

Extracorporeal membrane oxygenation (ECMO) has been widely used to provide cardiopulmonary support for neonates with severe respiratory failure.[1] Since 1985, more than 10,000 neonates have been treated with ECMO. Following cannulation of jugular vessels, hypoxemia is corrected by the reinfusion of well-oxygenated blood (PaO_2 > 300 mmHg) back to the systemic circulation. During ECMO, blood is exposed not only to hyperoxia but also a large artificial surface area (silicone membrane oxygenator and polyvinyl tubing) that can cause platelet activation.[2] Indeed, ECMO is associated with increased plasma levels of various cytokines and endotoxin.[3] Platelet activation during cardiopulmonary bypass can contribute to hemorrhagic and thromboembolic complications, including excessive post-operative bleeding.[4] While thrombocytopenia with abnormal platelet aggregatory function during ECMO has been reported,[5] the direct evidence and temporal changes of platelet activation are lacking.

Matrix metalloproteinases (MMPs) belong to a family of zinc neutral endopeptidases.[6] Among the MMPs, a 72-kDa matrix metalloproteinase-2 (MMP-2, gelatinase A) and a 92-kDa matrix metalloproteinase-9 (MMP-9, gelatinase B) are involved in the degradation of collagen (gelatin) and intact collagen type IV, a major component of the basement membrane.[6] The MMPs are released extracellularly and involved in the remodeling of the extracellular matrix in tissue during various physiological and pathological conditions such as embryonic development, inflammation, cancer and myocardial infarction.[7,8] Recently, Sawicki et al found MMP-2 in the releasate of activated human platelets and reported that this enzyme

mediates a novel pathway of platelet aggregation.[9] This provides evidence for the dual physiological role of MMPs; acutely as a regulator of platelet function, and chronically in the regulation of extracellular matrix remodeling. Tissue inhibitors of MMPs (TIMPs) are endogenous proteins that inhibit MMPs activity.[10] TIMP-2, a specific inhibitor of MMP-2,[11] binds to MMP-2 and has been shown to inhibit collagen-induced platelet aggregation.[9] Despite extensive research on tissue remodeling during pathological conditions in adults, there is no information on the role and changes of plasma MMP-2, MMP-9 and TIMP-2 in critically ill neonates.

I tested the hypothesis that following the initiation of ECMO platelets are activated and release MMP-2 which contributes to the platelet dysfunction. I therefore prospectively investigated the temporal changes of platelet aggregatory function in critically ill neonates treated with ECMO along with changes in the plasma MMP-2 and MMP-9 activities and TIMP-2 level during ECMO.

MATERIALS AND METHODS

The study was approved by the Research Ethics Board at the University of Alberta, Edmonton, Alberta, Canada.

Patients

Between September 1996 and March 1997, following informed parental consent, all 11 neonates requiring ECMO were enrolled in this study. One neonate was excluded because of incomplete data collection. Demographic and clinical features (birth weight, gestational age, primary diagnoses, highest oxygenation index (defined as inspired oxygen concentration x mean airway pressure (cm H_2O) x 100 ÷ arterial oxygen tension (mmHg)) and lowest arterial pH before initiation of ECMO were studied in the remaining 10 neonates. In our centre, ECMO was initiated when 3 oxygenation indices $\geq$ 40 occurred within a 2-hr window along with a mean airway pressure $\geq$ 18 cm H_2O. The protocol of management of ECMO in neonates has been previously described.[8,12]

Whole blood (3 ml) was collected in 3.15% tri-sodium citrate preparation (9:1 v:v) at defined intervals: within an hr prior to ECMO (pre-ECMO baseline), at 1, 2, 4, 12, and 24 hr following initiation of ECMO, and 1 hr before and 24 hr after discontinuation of ECMO. Whole blood aggregometry was tested within 15 min following collection. Platelet poor plasma (supernatant of whole blood after centrifugation at 760 g for 10 min at 4 °C) was prepared in aliquots and stored at -80 °C for subsequent biochemical analysis.

Platelet number and aggregation

Platelet number was measured using an automated platelet counter (Coulter MicroDiff 16, Hialeah, FL. USA).

Platelet aggregation was studied using a whole blood platelet-ionized Ca^{2+} lumi-aggregometer (Chrono-Log, Havertown, PA, USA). Citrated blood (495 µl) was diluted with an equal amount of sterile saline, stirred at 1000 rpm and incubated at 37 °C for 3 min prior to the addition of 10 µl collagen solution (to give final concentrations of either 2, 5 and 10 µg/ml, Chrono-Log, Havertown, PA, USA). Platelet aggregation was measured for 3 min and analyzed using the Aggro/Link computer data reduction system.

Matrix metalloproteinases activities in plasma

Gelatin-degrading activities of plasma were examined by zymography, which has been described previously,[9] using the protocol of Heussen and Dowdle.[13] Briefly, non-heated samples were mixed with SDS sample buffer without 2-mercaptoethanol and applied to 8 % polyacrylamide separating gels copolymerized with gelatin (2 mg/ml, Sigma). The serum-free medium conditioned by HT 1080 cells (American Type Culture Collection, Rockville, MD) which secrete large amounts of MMP-2, MMP-9 and TIMP-2 was used as standard control. HT 1080 cells were maintained at 37 °C in a 5 % CO_2 atmosphere in Eagle's MEM media with 10 % fetal calf serum at a concentration of 5 x 10^5 cells/ml. To collect cell-conditioned media, the cells were washed 3 times with serum-free media and incubated at 37 °C for 24 hr. In some

experiments the media were concentrated approximately 20-fold using a Centricon 10 concentrating vessel (Amicon Inc., Beverly, MA).

Plasma samples were subjected to electrophoresis, then the gels were washed three times with 2 % Triton X-100 to remove SDS and then incubated in incubation buffer (50 mM Tris-HCl buffer with 0.15 M NaCl, 5 mM $CaCl_2$ and 0.05 % NaN_3, pH 7.5) overnight to determine the activity of the enzymes. After incubation, the gels were stained with 0.05 % Coomassie Brilliant Blue G-250 (Sigma, St. Louis, MO) in a mixture of methanol: acetic acid: water (2.5: 1: 6.5, v:v) and de-stained in 4 % methanol with 8 % acetic acid. Gelatinolytic activities were detected as transparent bands against the background of Coomassie Brilliant Blue-stained gelatin. Prestained molecular weight standards of the 47-205 kDa range (Bio-Rad, Mississauga, ON) and the 15-217 kDa range (Gibco BRL, Burlington, ON) were used for reference. To quantitate the activities of the detected enzymes, zymograms were processed in the ScanJet 3c scanner (Hewlett Packard, Boise, ID). The intensities of the digitized bands were analyzed using SigmaGel measurement software (Jandel Corporation, San Rafael, CA). To confirm further the specificity of gelatinolysis, *o*-phenanthroline (20 μM), a specific inhibitor of MMPs, was added to the incubation buffer. Incubation was carried out overnight, followed by staining and analysis as described above.

Identification of TIMP-2 in plasma by Western blot

The plasma sample was applied to 12 % SDS-PAGE and electrophoresis was carried out under reducing conditions according to Laemmli's method.[14] After electrophoresis the sample was electroblotted onto polyvinylidene difluoride

membranes (Schleicher and Schulell, Keene, NH) and probed with monoclonal antibodies against TIMP-2 (5 µg/ml) (Oncogene Science, San Diego, CA). The detection of protein bands was carried out with an ECL detection kit (Amersham, Oakville, ON).

Measurement of plasma soluble P-selectin, soluble E-selectin, nitrates and nitrites during ECMO

Soluble P-selectin (sP-selectin) and soluble E-selectin (sE-selectin) in plasma were measured by enzyme immunoassays using kits from R & D Systems (Minneapolis, MN). For plasma NO_X^- measurement, plasma samples were deproteinated by ultrafiltration using a Centrifree micropartition system (Amicon). Samples were then analysed using an automated system according to the method of Green et al.[15] This method of detection is based on the nitrite reaction with Griess reagent to give a coloured product which could be detected at the visible wavelength of 546 nm. The nitrate content of the plasma sample is quantitatively reduced to nitrite as it passes through a cadmium precolumn before being mixed with the Griess reagent and then analysed on-line using a visible light absorbance detector.[15] The limit of detection is 1.0 µM NO_X^-.

Measurement of plasma lipid hydroperoxides during ECMO

Lipid hydroperoxides were extracted from plasma and assayed using a commercial colorimetric kit (Cayman Chemical Co., Ann Arbor, MI). The extraction procedure was combined with a deproteination step using methanol and chloroform.

The absorbance at 500 nm of the chloroform-methanol (1:1 v:v) mixture containing chromogen (ferric thiocyanate) was compared with standard solutions of 13-hydroperoxyoctadecadienoic acid (0.5-5 μM).

STATISTICS

Data are expressed as mean ± standard error of mean. The results were analysed by one-way ANOVA using Jandel SigmaStat 2.0 statistical software (Jandel Corporation, San Rafael, CA, USA). Fisher's least significant difference method was used for post-hoc testing. Correlation between variables was studied by the Pearson Product Moment test. Statistical significance was set at $P < 0.05$.

RESULTS

1. Patients

Ten neonates were studied, they weighed 3290 ± 220 (range 2200-4000) g at birth, were 39 ± 0.5 (range 36-41) weeks of gestation, and had either meconium aspiration syndrome (5) or congenital diaphragmatic hernia (5) as the primary diagnosis of severe respiratory failure. All had high oxygenation index: 98 ± 23 (range 38-267); low arterial pH: 7.23 ± 0.07 (range 6.79-7.61) and high plasma lactate levels: 10.1 ± 2.0 (range 5.2-24.6) mM before initiation of ECMO. Venovenous (8) or venoarterial (2) ECMO was initiated between 13 and 61 (25 ± 4.3) hr of postnatal age. The complete profile of platelet aggregatory function and biochemical data were collected for all but 2 neonates of whom only the first 24 hr data were collected. The latter 2 neonates died following withdrawal of ECMO therapy (severe intracranial hemorrhage and multiple organ failure, respectively). The duration of ECMO therapy was between 31 and 188 (91 ± 16) hr.

2. Platelet dysfunction during ECMO

These neonates had platelet counts of 132 ± 17 (range 49-212) x 10^9/L before initiation of ECMO. Within 1 hr after initiation of ECMO, platelet counts decreased by 47 ± 7 % from pre-ECMO values (Fig. 6.1A). Platelet concentrates (70 ± 11 ml) were given in the first 24 hr after initiation of ECMO and continued until 6 hr before discontinuation of ECMO to maintain platelet counts > 100 x 10^9/L. Despite the platelet transfusion, the aggregatory response upon collagen stimulation (10 µg/ml) was significantly inhibited during the first 24 hr after initiation of ECMO (Fig. 6.1B).

Thereafter, the platelet aggregatory function gradually improved but remained modestly inhibited before discontinuation of ECMO (74 ± 15 % of pre-ECMO baseline). At 24 hr after discontinuation of ECMO the extent of platelet aggregation was not different from pre-ECMO baseline, despite of significant thrombocytopenia ($76 \pm 7 \times 10^9$/L vs. pre-ECMO baseline, $P < 0.05$) (Fig. 6.1B). In response to collagen stimulation at 2 and 5 μg/ml, platelet aggregation was significantly inhibited (20 ± 5 and 31 ± 6 % of pre-ECMO baseline at 1 hr after initiation of ECMO, respectively) and followed a trend similar to that of 10 μg/ml collagen-induced response.

3. Plasma MMP-2, MMP-9 and TIMP-2 levels during ECMO

Gelatinolytic activities of MMP-2 and MMP-9 in the plasma of these neonates are shown on the zymogram in Fig. 6.2A. After initiation of ECMO, plasma MMP-2 activity progressively increased and became significantly elevated at 12 hr after initiation of ECMO (Fig. 6.2B). The peak increase in plasma MMP-2 activity occurred at 24 hr after initiation of ECMO and was approximately 2-fold (210 ± 12 %) of the pre-ECMO baseline. Plasma MMP-2 activity at 24 hr after discontinuation of ECMO was similar to the pre-ECMO activity.

In contrast, plasma MMP-9 activities were not significantly altered when compared with pre-ECMO activity (Fig. 6.2C).

By Western blot analysis, TIMP-2 in the plasma samples collected from these neonates throughout the study period was below the detection limit (data not shown).

4. Plasma sP-selectin, sE-selectin and NO_X^- levels during ECMO

Plasma levels of sP-selectin progressively increased after initiation of ECMO and became significantly elevated at 4 hr after initiation of ECMO (Fig. 6.3A). Before discontinuing from ECMO, plasma sP-selectin level increased by almost 2-fold (194 ± 19 %) compared with pre-ECMO baseline of 110 ± 4 ng/ml. Plasma sP-selectin returned to pre-ECMO baseline by 24 hr after discontinuation of ECMO. A significant linear correlation was found between plasma levels of sP-selectin and MMP-2 ($r = 0.61$, $P < 0.001$, $n = 37$), but not between sP-selectin and MMP-9 ($r = -0.17$, $P > 0.05$, $n = 35$).

During ECMO, the plasma level of sE-selectin ranged from 49 ± 5 to 67 ± 9 ng/ml. In contrast to the progressive increase in sP-selectin, there were no significant changes found in the plasma levels of sE-selectin during ECMO.

Plasma NO_X^- levels were not significantly altered during ECMO (Fig. 6.3B).

5. Plasma lipid hydroperoxides during ECMO

Before initiation of ECMO, the plasma level of lipid hydroperoxides was 5.0 ± 0.6 μM. Plasma lipid hydroperoxides gradually increased after initiation of ECMO, and became maximal at 2 hr after initiation of ECMO (6.5 ± 1.2 μM, 25 ± 7 % increase from pre-ECMO baseline, $P < 0.05$). Thereafter, the plasma levels of lipid hydroperoxides remained significantly higher than pre-ECMO baseline until before the discontinuation of ECMO (Fig. 6.3C). After discontinuing ECMO, there were no further changes in plasma lipid hydroperoxides level.

DISCUSSION

I have shown direct evidence for platelet activation and dysfunction in neonates treated with ECMO. In addition, I have described the time-course of these pathological changes. Platelet aggregation was significantly depressed during the first 24 hr of ECMO despite repeated platelet transfusions to maintain platelet counts > 100 x 10^9/L. Although volume dilution due to the initiation of ECMO may contribute to the thrombocytopenia and platelet dysfunction, the absence of correlation between platelet numbers and aggregation suggest other mechanisms are responsible for the dysfunction. The short-lived platelet function, which is consistent with that observed by Robinson et al,[5] may partially explain the occurrence of haemorrhagic complications in the presence of adequate platelet count and coagulatory function.

I have demonstrated that ECMO results in an increase in sP-selectin, but not sE-selectin levels in plasma. P-Selectin is a 62-kDa cell surface glycoprotein that plays a critical role in the adhesion of platelets to monocytes and neutrophils.[16] P-Selectin is stored in the alpha granules of platelets and in the Weibel-Palade bodies of endothelial cells.[17] Upon activation, the stored P-selectin is mobilised to the cell surface within min and then recycled to intracellular compartments later. The circulating P-selectin (sP-selectin) appears to be slightly smaller than native P-selectin.[17] It appears that platelets are the major source of circulating sP-selectin in human and this makes the protein a marker of platelet activation in vivo.[18] E-Selectin (endothelial leukocyte adhesion molecule-1) is a 115-kDa cell surface glycoprotein that is transiently expressed on vascular endothelial cells in response to IL-1 and TNF-α.[19] E-Selectin also exists in a soluble, non-membrane associated form that has

been found to be elevated in inflammatory conditions such as septic shock.[20] While the expression of E-selectin is restricted to endothelial cells, levels of soluble E-selectin (sE-selectin) are believed to represent a specific marker for endothelial damage or activation that occurs during cardiopulmonary bypass.[21] A differential increase of sP-selectin vs. sE-selectin during ECMO suggests that platelet but not endothelial activation is the major source of sP-selectin. This is also supported by the absence of significant changes in plasma NO_X^- levels, which argues against endothelial activation. Platelets are activated during ECMO because of the artificial surfaces.[2] However, the hyperoxic environment with the generation of free oxygen radicals may also contribute to the platelet activation.[9] Consistent with the report by Hirthler et al,[3] the progressive increase in plasma level of lipid hydroperoxides suggests the presence of oxidative stress during ECMO.

In addition, plasma sP-selectin levels correlated well with MMP-2 activities. MMP-2 has been recently shown to regulate platelet aggregation,[9] in addition to its role in the extracellular matrix remodelling during various physiological and pathological conditions.[6] Indeed, low levels of MMP-2 stimulate platelet aggregation whereas high levels exert an inhibitory effect.[9] These actions of MMP-2 could be important in the pathogenesis of thrombotic and haemorrhagic complications in critically ill neonates, including those treated with ECMO as shown in this study. While the source of plasma MMP-2 remains to be investigated, impaired hepatic clearance is not likely to be the cause of elevated plasma MMP-2 because all of these neonates had normal liver function and serum bilirubin levels during ECMO. However, I cannot exclude the possibility that MMP-2 was released from other cell

types, including leukocytes and stromal cells.

Furthermore, I did not detect any significant amount of TIMP-2 in the plasma of these neonates using Western blot, which is far less sensitive than zymography in detecting protein. While cautious interpretation of this finding may be necessary, if plasma TIMP-2 levels indeed reflect tissue concentrations, this may pose important consequences to the unbalanced pathological effect due to elevated MMP-2 levels, which may contribute to the platelet dysfunction and increase the risk of haemorrhagic complications. The amount of other tissue inhibitors of MMPs such as TIMP-1 and TIMP-3, which cross-react with MMP-2, needs to be investigated.

Therapeutic interventions to alleviate platelet dysfunction during ECMO should be directed to prevent or inhibit the platelet activation. Nitric oxide (NO), prostaglandins and MMPs are important regulators of platelet aggregatory function.[9,22] Preliminary reports have shown that NO supplementation is effective in reducing the platelet activation.[23,24] Mellgren et al have demonstrated higher platelet counts in experimental perfusion circuits treated with sweep gas containing NO gas (15-75 ppm).[23] However, platelet function was not measured in their study. Annich et al coated the circuit polypropylene tubes with a NO adduct that releases NO upon reaction with water vapour and showed less platelet adhesion to the circuit surface and less platelet destruction during ECMO in rabbits.[24] Recently, Radomski et al found that inhibition of NO synthesis caused an enhancement of MMP-2 activity in the lungs of rats with hyperoxic lung injury.[25] This compliments the current findings that new therapeutic interventions including NO supplementation can modulate MMPs activity and may be useful to alleviate platelet activation during ECMO.

In this study, MMP-2 was released upon platelet activation during ECMO and might have contributed to the platelet dysfunction. I believe that the role of MMP-2 in ECMO is significant and could be generalised to other clinical conditions involving extracorporeal circulation such as cardiopulmonary bypass and hemodialysis, which also demonstrate platelet activation and dysfunction resulting in thrombotic and haemorrhagic complications.

Fig. 6.1 Thrombocytopenia and inhibited platelet aggregatory function during extracorporeal membrane oxygenation (ECMO).

Thrombocytopenia and depressed aggregatory response to collagen (10 μg/ml) stimulation were measured by whole blood aggregometry within 1 hr after initiating ECMO. Platelet function remained significantly below the pre-ECMO baseline values during the first 24 hr of ECMO, although repeated platelet concentrate transfusions were given. Despite thrombocytopenia, platelet function returned to near pre-ECMO values at 24 hr after discontinuation of ECMO.

Pre: within 1 hr initiation of ECMO, Pre-D/C: within 1 hr before discontinuation of ECMO.

* $P < 0.05$ vs. pre-ECMO baseline values (n = 8-10, ANOVA).

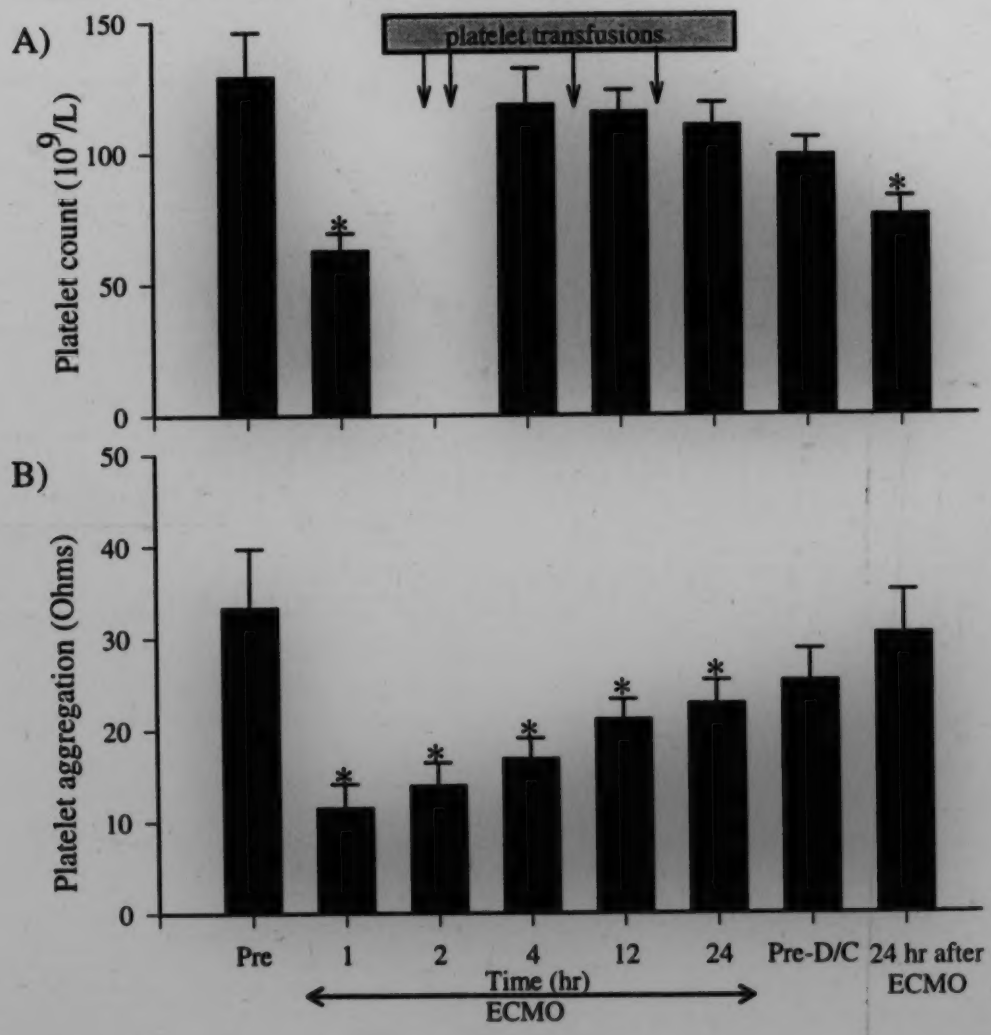


Fig. 6.2 Elevation of plasma matrix metalloproteinase-2 (MMP-2) but not MMP-9 activities during extracorporeal membrane oxygenation (ECMO).

(A) A representative zymogram from one neonate showing plasma gelatinolytic MMP activities. (B) Quantification of relative intensities (arbitrary density units) of MMP-2 activity in plasma of neonates shows a maximal increase at 24 hr after initiating ECMO. (C) Quantification of relative intensities (arbitrary density units) of MMP-9 activity in plasma of neonates shows no significant changes during ECMO.

Pre: within 1 hr initiation of ECMO, Pre-D/C: within 1 hr before discontinuation of ECMO.

* $P < 0.05$ vs. pre-ECMO baseline values (n = 8-10, ANOVA).

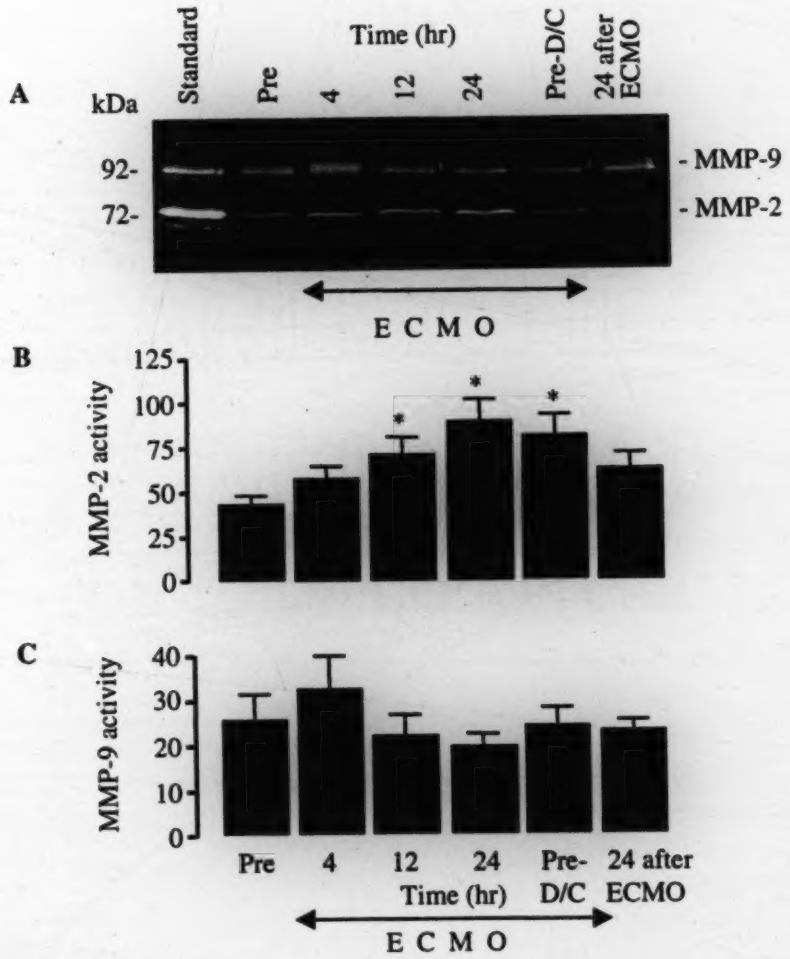
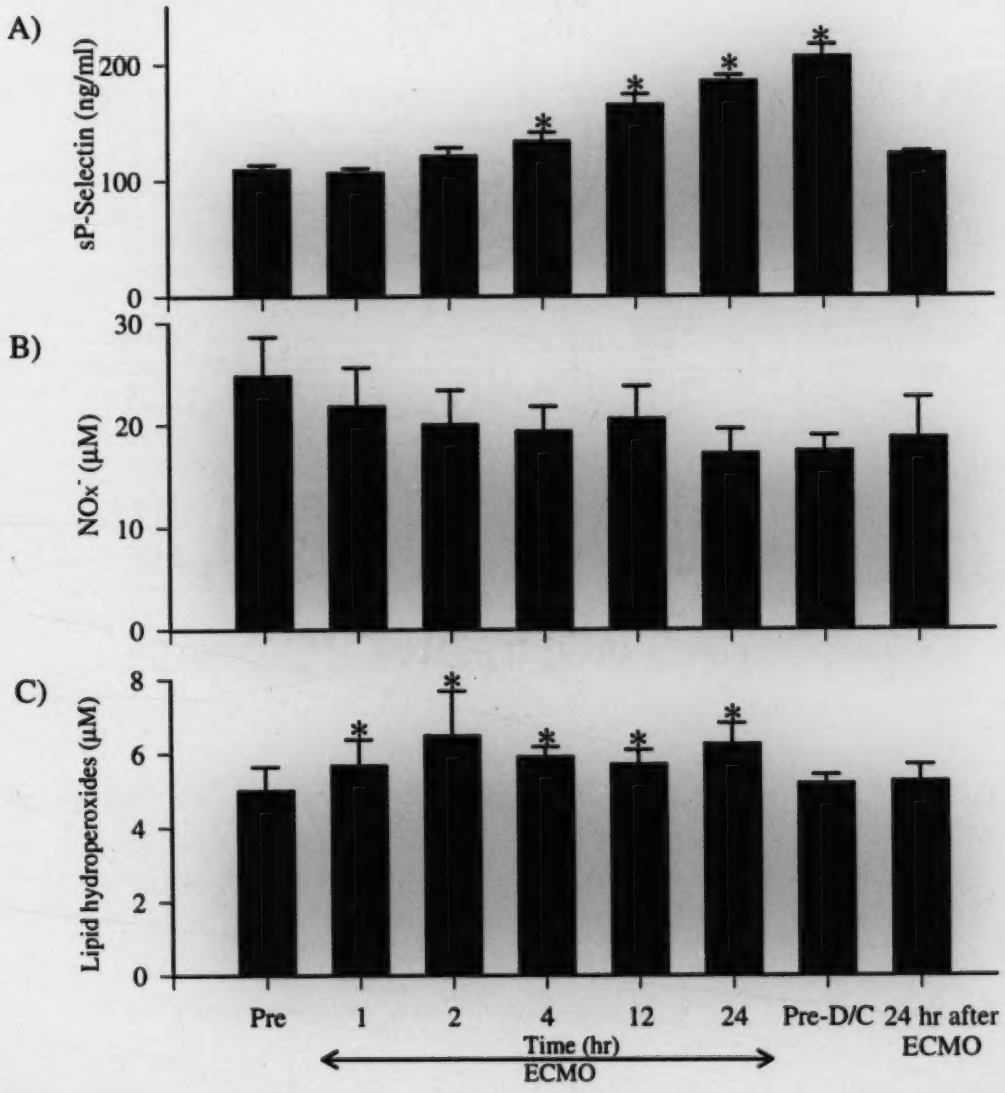


Fig. 6.3 Plasma levels of (A) sP-selectin, (B) nitrite and nitrate (NO_X^-) and (C) lipid hydroperoxides before, during and after extracorporeal membrane oxygenation (ECMO).

Plasmå sP-selectin progressively increased after initiating ECMO, became maximum before discontinuation of ECMO and returned to near pre-ECMO values 24 hr after discontinuation. No significant changes in plasma NO_X^- levels were detected during ECMO whereas lipid hydroperoxides were significantly elevated during the first 24 hr of ECMO.

Pre: within 1 hr initiation of ECMO, Pre-D/C: within 1 hr before discontinuation of ECMO.

* $P < 0.05$ vs. pre-ECMO baseline values (n = 8-10, ANOVA).



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CHAPTER VII

INHALED NITRIC OXIDE INHIBITS PLATELET AGGREGATION IN CRITICALLY ILL NEONATES

A version of this chapter has been published. Cheung P-Y, Salas E, Etches PC, Phillipos E, Schulz R, Radomski MW. (1998) Lancet 351:1181-1182.

ABSTRACT

Although inhaled nitric oxide (NO) causes inhibition of platelet function and prolongation of bleeding time in adults, its effect on platelets of critically ill neonates is unknown. I have, therefore, studied the effect of inhaled NO (2-80 ppm) on platelet aggregation in mechanically ventilated neonates (n = 14). This effect was compared with those in mechanically ventilated (n = 10) and non-mechanically ventilated (n = 7) neonates who did not receive inhaled NO. Collagen induced platelet aggregation was measured in whole blood using impedance aggregometry. Inhaled NO resulted in a significant inhibition of aggregation that was associated with elevated cyclic GMP levels in platelets. Some neonates had persistently inhibited platelet aggregation at 6 to 24 hr following the discontinuation of inhaled NO. I conclude that caution should be taken with the administration of inhaled NO to critically ill neonates, as inhibition of platelet aggregation by NO may increase the risk of intracranial haemorrhage.

INTRODUCTION

In the vasculature, generation of nitric oxide (NO) regulates vascular wall reactivity and platelet function[1] primarily through the stimulation of soluble guanylate cyclase. As some vascular disorders are associated with decreased NO bioactivity, the pharmacological supplementation of endogenous NO with NO donors or inhaled NO gas is an attractive therapeutic strategy in these disorders.[2]

The concept of selective pulmonary vasodilation following inhaled NO[3] is based on its direct access to the pulmonary vasculature and a very short half-life of NO gas in biological milieu[4] that apparently precludes its effects on the systemic circulation. Indeed, inhaled NO has been shown to cause selective pulmonary vasodilation and improve oxygenation in critically ill neonates with a low incidence of systemic side effects such as methemoglobinemia.[5,6] However, there are reports showing that inhaled NO prolongs bleeding time and inhibits platelet aggregation in adults,[7,8] thus increasing the risk of haemorrhagic complications.

In critically ill neonates, intracranial haemorrhage is a serious complication that can increase mortality and affect subsequent neurodevelopmental morbidity of survivors. This prospective study was, therefore, designed to investigate the effect of inhaled NO on platelet aggregation in critically ill neonates.

MATERIALS AND METHODS

In our centre, inhaled NO is administered at the discretion of the attending neonatologist, following informed parental consent. Inhaled NO is administered to treat severe hypoxemia unresponsive to aggressive conventional therapy including surfactant, high frequency ventilation and inotropic support. The concentration of inhaled NO is started at 20 ppm and adjusted according to the clinical response as previously described.[6]

Between August 1996 and July 1997, following informed parental consent, three groups of neonates were prospectively enrolled into the study: group I – critically ill neonates treated with inhaled NO (2-80 ppm), group II – critically ill neonates who were mechanically ventilated but not treated with inhaled NO (ventilated controls), and group III - neonates who were admitted to the neonatal intensive care unit for mild respiratory problems, such as transient tachypnea of newborn and who were not mechanically ventilated (non-ventilated controls). All these neonates had arterial catheters inserted for blood sampling. Neonates receiving indomethacin (a known inhibitor of platelet aggregation) were not enrolled in this study.

Demographic and clinical features (gestational age, birth weight, Apgar scores at 1 and 5 min, primary diagnoses and the requirement for extracorporeal membrane oxygenation) were recorded. In addition, highest oxygen index (defined as inspired oxygen concentration x mean airway pressure (in cm H_2O) x 100 ÷ arterial oxygen tension (in mmHg)) and lowest arterial pH were studied in groups I and II.

In group I, blood samples for measuring platelet aggregation were collected at 4 hr following the initiation of inhaled NO. In this group, platelet aggregation was also

studied at 6 and 24 hr following the discontinuation of inhaled NO, except in those neonates who subsequently required extracorporeal membrane oxygenation due to persistent hypoxemia. For groups II and III, platelet aggregation was measured within 72 hr after birth. Platelet counts and intraplatelet cyclic GMP levels were assayed in all groups. In group I, blood methemoglobin content was measured every 8 hr by co-oximeter (IL 482 CO-Oximeter, Instrumentation Laboratory Co., Lexington, MA, USA). Cranial sonography was performed before discharge for all group I neonates.

Measurement of platelet aggregation

1.8 ml of blood was collected into tubes containing 0.2 ml of 3.15% (w:v) tri-sodium citrate. Within 10 min following blood collection, platelet aggregation was studied using a whole blood platelet-ionized Ca^{2+} lumi-aggregometer (Chrono-Log, Havertown, PA, USA). Blood (495 µl) was diluted with an equal amount of sterile saline, stirred at 1000 rpm and incubated at 37 °C for 3 min prior to the addition of 10 µl collagen (2, 5 and 10 µg/ml, Chrono-Log, Havertown, PA, USA). Platelet aggregation was measured for 3 min and analysed using the Aggro/Link computer data reduction system.

Measurement of intraplatelet cyclic GMP levels

3-Isobutyl-1-methylxanthine (100 µM) was added to the citrated blood (0.5 ml) to inhibit the activity of cyclic nucleotide phosphodiesterases. The samples were centrifuged (220 g for 20 min at room temperature) to obtain platelet rich plasma. Platelets were separated from platelet rich plasma by centrifugation at 760 g for 10 min at 4 °C. The resultant pellet was stored until assayed at -80 °C. For cyclic GMP

assay, pellets were homogenised in a buffer (50 mM Tris buffer containing 320 mM sucrose, 1 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 100 µM 3-isobutyl-1-methylxanthine and 2 µg/ml aprotinin) by two cycles of freezing in liquid nitrogen and thawing at 37 °C. The homogenate was centrifuged (10,000 g, 20 min, 4 °C) and the supernatant used for the measurement of the intraplatelet cyclic GMP levels using dual range acetylation enzyme immunoassay (Amersham, Oakville, ON) according to the manufacturer's recommendations.

STATISTCAL ANALYSIS

Data are expressed as mean ± standard error of mean of n determinations. Effective index (EI_{50}) values refer to concentrations of collagen that induced half-maximal platelet aggregation assuming 10 μg/ml collagen caused maximal aggregation. One-way ANOVA with Fisher's least significant difference post-hoc testing, Student's *t* and paired *t* tests were used for the analysis of differences between groups and paired samples of continuous variables as appropriate. For categorical variables, Chi Square test was used. Jandel SigmaStat 2.0 statistical software (Jandel Corporation, San Rafael, CA, USA) was used. Statistical significance was set at $P < 0.05$.

RESULTS

Thirty-one neonates were enrolled in the study (Table 7.1). No significant differences were found between the groups in regard to birth weight, gestational age, Apgar scores at 1 and 5 min, platelet counts, highest oxygen index and lowest arterial pH and the requirement for extracorporeal membrane oxygenation (Table 7.1). The contribution of hyaline membrane disease, meconium aspiration syndrome, congenital diaphragmatic hernia, group B septicemia and other diseases to hypoxemia in each group were not significantly different from each other (Chi Square test, Table 7.1).

No significant difference in platelet aggregation was found between groups II and III (Fig. 7.1A). However, in group I, there was a significant inhibition of collagen-induced platelet aggregation as shown by right shifting of the concentration-response curve (Fig. 7.1A) and increased EI_{50} (Fig. 7.1B) of this group when compared with groups II and III. The inhibition of platelet aggregation by inhaled NO was associated with significantly higher cyclic GMP levels in platelets (Fig. 7.1C). I also studied the platelet function in 6 Group I neonates before and after NO inhalation. At 4hr of NO inhalation, the extent of aggregation was inhibited when compared with before NO values (paired *t* test, $P < 0.05$). This was demonstrated by a rightward shift of the concentration-response curve (Fig. 7.2A) and an increased EI_{50} (effective index-concentration of collagen causing half-maximal aggregation) (Fig. 7.2B).

During inhaled NO therapy, the peak methemoglobin levels were 2.3 ± 0.3 % (range: 1.5-3.4 %).

In group I, 6 out of 14 neonates subsequently required extracorporeal membrane oxygenation due to persistent hypoxemia. In the remaining 8 neonates, platelet aggregation following the discontinuation of inhaled NO was studied. The duration of inhaled NO therapy in these 8 neonates was 7-69 hr. The clinical data of these neonates are shown individually in Table 7.2. Four term neonates showed normal platelet aggregation (as defined in the legend to Table 7.2) at 6 and 24 hr following the discontinuation of inhaled NO. In contrast, platelet aggregation of the other 4 neonates was inhibited for as long as 6 hr following the discontinuation of inhaled NO. Moreover, platelet aggregation of neonates #6 and #8 remained persistently inhibited at 24 hr following the discontinuation of inhaled NO. Platelet aggregation of neonates #1 and #4 normalised at 24 hr. Both premature infants (#1 and #6), who also had severe metabolic acidosis and high oxygen indices, developed serious intraventricular haemorrhage later.

DISCUSSION

To my knowledge this is the first report examining the effects of inhaled NO on platelet aggregation in critically ill neonates. I have shown that inhaled NO inhibited platelet aggregation that was associated with elevated intraplatelet cyclic GMP levels. This is consistent with observations in adult volunteers[7] and patients with adult respiratory distress syndrome,[8] which showed that inhaled NO inhibited platelet function. My findings also suggest that the platelet-inhibitory effects of inhaled NO depend on increased generation of cyclic GMP in platelets of neonates.

In addition, I found that the inhibition of platelet aggregation persisted in some critically ill neonates even when inhaled NO had been discontinued for 24 hr. Hogman et al first reported a prolongation of bleeding time for approximately 30 min following the discontinuation of NO inhalation in healthy volunteers.[7] In their preliminary report of neonates treated with inhaled NO, George et al also noted a prolonged bleeding time one hr after inhaled NO had been discontinued.[9]

The mechanism of the prolonged effect of inhaled NO on platelet function may be explained by the accumulation and subsequent slow release of NO from endogenous storage forms such as *S*-nitrosothiols.[10] This hypothesis is supported by a recent report of a second peak of serum nitrate and nitrite concentrations 3 hr following the discontinuation of inhaled NO in human volunteers.[11] Poor oxygenation, altered antioxidant balance,[12] changes in metabolism of NO and increased sensitivity of foetal platelets to inhibition by NO[13] could also account for prolonged effects of inhaled NO on platelets of critically ill neonates.

Interestingly, this long-lasting inhibition of platelet aggregation was detected in

2 premature neonates with severe metabolic acidosis and hypoxemia. These neonates subsequently developed serious intraventricular haemorrhage. Thus, it is possible that inhaled NO in premature neonates may cause prolonged impairment of platelet function and increase the risk of intracranial haemorrhage. Intracranial haemorrhage can affect mortality and neurodevelopmental outcome in critically ill neonates, especially in premature neonates.[14] Van Meurs et al have already expressed the concern about the high incidence of intracranial haemorrhage in premature neonates treated with inhaled NO.[15] My results support this concern and provide a rationale for inhibition of platelet hemostasis by inhaled NO in critically ill neonates.

Despite its effectiveness in the improvement of systemic oxygenation, inhaled NO is still being considered for approval by the US Food and Drug Administration. Until the balance of benefits and risks of this therapy is clearly assessed, caution is advised for the administration of inhaled NO, and careful patient selection criteria should be developed before subjecting critically ill neonates to inhaled NO.

Table 7.1 Clinical data in neonates treated with inhaled NO and in ventilated and non-ventilated controls.

	Group I inhaled NO (n = 14)	Group II ventilated controls (n = 10)	Group III Non-ventilated controls (n = 7)
Birth weight (g)	2964 ± 277	2434 ± 385	2496 ± 350
Gestation age (weeks)	37.8 ± 1.3	34.6 ± 1.7	34.6 ± 1.8
Apgar score at 1 min	4.8 ± 0.7	4.5 ± 0.6	4.9 ± 0.9
Apgar score at 5 min	6.8 ± 0.5	5.7 ± 0.7	6.3 ± 0.7
Platelets (10^6/L)	158 ± 17	189 ± 18	182 ± 27
Highest oxygen index	55 ± 9	45 ± 12	Not applicable
Lowest arterial pH	7.19 ± 0.05	7.22 ± 0.03	Not applicable
Number requiring ECMO	6	4	Not applicable
Primary Diagnosis:			
HMD	3	4	5
MAS	4	2	1
CDH	4	4	0
GBS	2	0	0
Others	1	0	1

Legends:

oxygen index = inspired oxygen concentration x mean airway pressure (in cm H_2O) x 100 ÷ arterial oxygen tension (in mmHg)

ECMO = extracorporeal membrane oxygenation

HMD = hyaline membrane disease, MAS = meconium aspiration syndrome,

CDH = congenital diaphragmatic hernia, GBS = Group B septicemia,

Others: persistent pulmonary hypertension of newborn in Group I, and congenital heart disease in Group III.

Table 7.2 Clinical data and platelet aggregation at 6 and 24 hr following the discontinuation of inhaled NO to 8 neonates.

	BW (g)	GA (wk)	Dx	OI	pH	Plat (10^6/L)	Platelet aggregation 6 hr	24 hr	US Head
1	1220	29	HMD	52	6.97	143	↓	N	IVH1
2	2600	41	PPHN	18	7.29	120	N	N	--
3	3625	41	CDH	6	7.27	152	N	N	--
4	2850	40	MAS	34	7.29	118	↓	N	--
5	3800	39	GBS	18	7.52	245	N	N	--
6	630	24	HMD	37	7.05	107	↓	↓	IVH2
7	2410	37	HMD	45	7.43	201	N	N	--
8	3760	40	GBS	65	7.31	62	↓	↓	--

Legends:

BW = birth weight, GA = gestational age, Dx = primary diagnosis, OI = highest oxygen index, pH = lowest arterial pH, Plat = lowest platelet count.

HMD = hyaline membrane disease, PPHN = persistent pulmonary hypertension of newborn, CDH = congenital diaphragmatic hernia, MAS = meconium aspiration syndrome, GBS = Group B septicemia.

N = normal platelet aggregation: this was within the range (mean ± two standard deviations) of responses induced by 10 μg/ml collagen in groups II and III, ↓ = inhibited platelet aggregation that was outside this range.

US Head = cranial sonography results, IVH1 = intraventricular hemorrhage with bilateral dilatation of ventricles and IVH2 = intraventricular hemorrhage with bilateral parenchymal extension.

Fig. 7.1 Inhaled NO inhibited platelet aggregation and increased cyclic GMP levels in neonates.

(A) The concentration-response curves of collagen-induced platelet aggregation in whole blood. (B) Effective index (EI_{50}) values. (C) The intraplatelet cyclic GMP levels.

Group I: 14 neonates treated with inhaled NO, Group II: 10 ventilated controls, and Group III: 7 non-ventilated controls.

(A) * : group I vs. group III; # : group I vs. group II ($P < 0.05$, one-way ANOVA)

(B) and (C) * $P < 0.05$ (one-way ANOVA)

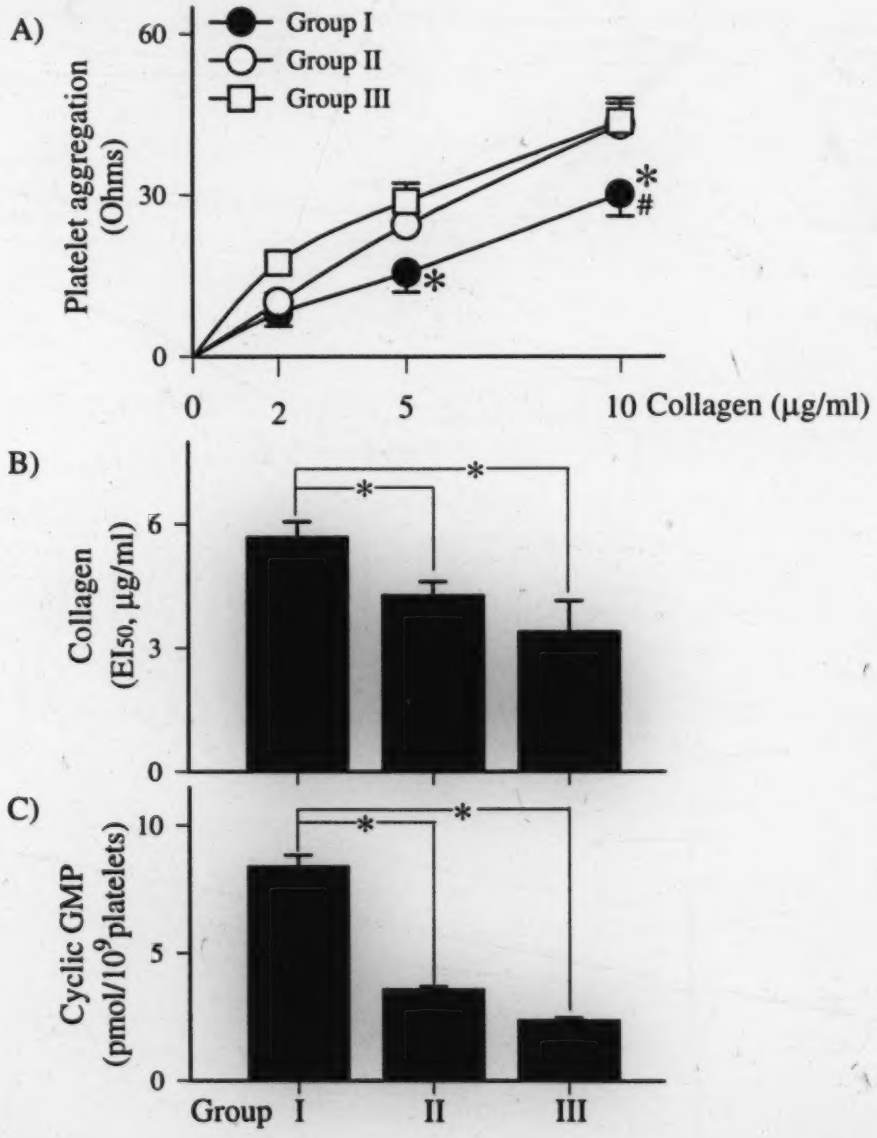


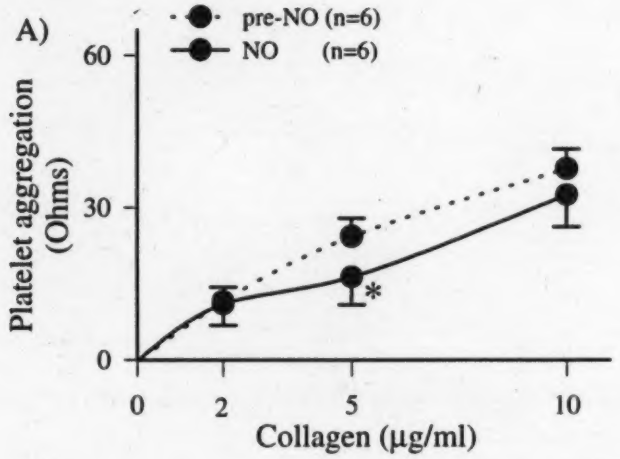
Fig. 7.2 Inhibition of platelet aggregation in 6 neonates after NO inhalation.

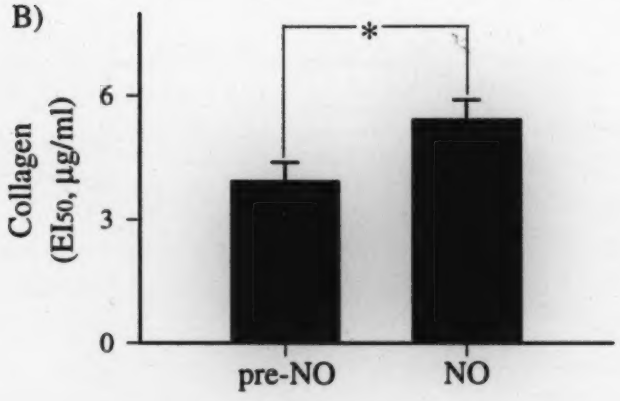
A. The concentration-response curves of collagen-induced platelet aggregation in blood.

B. Effective index (EI_{50}) values.

Before NO and NO data were obtained before and 4 hr after inhalation of NO in neonates.

* $P < 0.05$ (paired *t* test)





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CHAPTER VIII

LIMITATIONS, CONCLUSIONS & FUTURE DIRECTIONS

LIMITATIONS

Caution is required to extrapolate the results from isolated heart experiments to in vivo conditions. While there is a lack of interaction of the heart with the vasculature and other systems in the body, the isolated heart perfusion model allows pharmacological studies of the heart including coronary vasculature and myocardium. Also, one has to be careful in comparing the findings in crystalloid-perfused hearts to those of blood-perfused hearts. Blood contains erythrocytes, leukocytes, platelets and plasma, which all have significant effect on heart functions as well as in the study of NO and $ONOO^-$. For example, hemoglobin in erythrocytes reacts with NO and albumin in plasma reacts with $ONOO^-$, and thus alter their vascular and cardiac protective or detrimental effects of the studied agent. Ischemia-reperfusion injury is associated with platelet and leukocyte activation,[1] and the use of crystalloid perfusate precludes the study of delayed myocardial stunning due to platelet and leukocyte activation. Moreover, the perfusion pressure (60 mmHg) used in my experiments was lower than the physiological pressure (100 mmHg). Although Langendorff suggested this perfusion pressure based on histological, metabolic and structural factors,[2] cautious interpretation of the results is required. Also, basal NO production could have been enhanced due to higher coronary flow in this model[3] and this might decrease coronary vascular tone and blunt the effect of vasodilators.

Hearts were perfused at constant pressure, instead of constant flow, in Langendorff fashion. Perfusion at constant pressure is preferred because whenever possible, mean perfusion pressure is maintained constant in the intact circulation.[2] However, as opposed to the constant flow method, coronary flow may change

following the administration of drugs. This may lead to a small change in the concentration of the administered drug, which was prepared based on the assumption of an average coronary flow of 14 ml/min. Moreover, I did not study a complete concentration-response curve in the analysis of potency and efficacy of some pharmacological agents (thiols, chapters 2, 3 and 4; *o*-phenanthroline, chapter 5). This is because of: (1) the choice of physiologically relevant concentrations, (2) adverse effects including decreased cardiac function at high concentrations, and (3) the limited solubility of certain reagents in a buffer without significant effect on final perfusate pH. Also, in the clinical study of inhaled NO (chapter 7), incomplete concentration-response curves of collagen (2-10 μg/ml) on whole blood aggregation were constructed because small amounts of blood (3 ml) were collected from neonates who have small circulatory blood volumes (75-85 ml/kg). Indeed, 10 μg/ml collagen is used in most platelet aggregation studies for maximal stimulation. Thus I could only compare the relative effectiveness, but not potency, between pharmacological agents. Given that the pharmacological maximal effects (efficacy) were not studied, apparent EC_{50} (chapter 3) and EI_{50} (effective index, chapter 7) values were calculated based on the assumption that the highest concentrations used in the studies had induced maximal responses. Understanding these limitations, some caution is necessary in the interpretation of the data, yet the use of relative effectiveness is justified and allows meaningful conclusions and implications.

In the study of the acute role of matrix metalloproteinase-2 (MMP-2) in hearts subjected to ischemia-reperfusion, I was unable to identify the source or transport mechanism for the release of pro MMP-2 and MMP-2 into coronary circulation from

the heart. The information may be useful in designing pharmacological agents to inhibit MMP-2 activity. Okamoto had shown the activation of MMPs of stimulated neutrophils by $ONOO^-$ in the absence of any change in the molecular weight or size of the enzyme.[4] While this may suggest a conformational change and subsequent activation of the enzyme, zymographic examination cannot differentiate whether the 72-kDa band represented a pure population of pro MMP-2 or a mixed population of pro MMP-2 and active MMP-2. Moreover, the experiment with *o*-phenanthroline is limited by the cardiac depressive action of this drug. Indeed, the currently available inhibitors of MMP-2 including *o*-phenanthroline are far from selective in their inhibition of MMP-2. They are basically zinc chelators,[5] which may also inhibit other zinc-containing enzymes such as superoxide dismutase. They also have limited selectivity for chelation of zinc over other divalent cations such as Ca^{2+}, thus some of their effects at high concentrations may be due to reduced Ca^{2+} levels.

In clinical studies confounding factors such as hemodynamic and oxygenation parameters of patients hinder the study of mechanisms of MMP-2- and inhaled NO-induced platelet dysfunction. Also, studies of complete concentration-response curves and the profile of aggregatory responses induced by collagen and other agents such as ADP can provide more information in understanding platelet dysfunction in these patients and explain at least in part the discrepant reports of inhaled NO on platelet function.[6] Although I shared the concern that the use of inhaled NO may cause or exacerbate intraventricular hemorrhage[7] because of its inhibitory effect on platelet aggregation, the small sample size also precludes me from analyzing the important relationship between intraventricular hemorrhage and platelet dysfunction.

CONCLUSIONS

Antioxidant treatments have been tried as myocardial protective agents against reperfusion or reoxygenation injury with variable success. I have demonstrated that glutathione (GSH) is a novel coronary vasodilator in aerobically perfused rat hearts. Moreover it improves the recovery of myocardial mechanical function during reperfusion of ischemic rat hearts. GSH is a unique sulfhydryl-containing antioxidant that can scavenge $ONOO^-$. The reaction between GSH and $ONOO^-$ results in the formation of disulfide and *S*-nitrosoglutathione which stimulates soluble guanylate cyclase and increases cyclic GMP levels,[8] which may in part mediate the vasodilator effect and protective action of GSH against myocardial stunning. I also showed GSH protects against $ONOO^-$-induced inhibition of myocardial aconitase in heart homogenates. Together, these findings suggest that GSH supplementation could be a novel therapy for coronary ischemia and myocardial protection against reperfusion or reoxygenation injury.

MMP-2 has dual physiological and pathological roles. Chronically, it plays a key role in the process of tissue remodeling following myocardial infarction[9] and congestive heart failure.[10] Acutely, it is a novel mediator of platelet aggregation.[11] I also found that MMP-2 (both pro-enzyme and active form) is the most abundant gelatinolytic enzyme released acutely into coronary circulation during normal aerobic perfusion and whose release is enhanced during reperfusion following ischemia. Although the mechanism of MMP-2 in the regulation of cardiac function has not been determined, the role of MMP-2 in reperfusion injury could be significant because: (1) the release of MMP-2 is associated with myocardial stunning, and (2) specific

inhibition of MMP-2 activity results in the improvement of myocardial function during reperfusion. Indeed, MMP-2 may also be important in the impaired recovery of myocardial function later in reperfusion as a result of platelet and leukocyte activation,[1] which was not evaluated in these experiments using the isolated rat heart perfused with cell-free crystalloid perfusate. Inhibitors of MMP-2 may be a novel therapy for myocardial stunning injury.

I have delineated the time-course and mechanism of platelet dysfunction during extracorporeal membrane oxygenation (ECMO) and inhaled NO therapy. Both of these novel interventions have been used to rescue neonates with life-threatening diseases.[12,13] The platelet dysfunction in the presence of adequate platelet number during ECMO is due to platelet activation, which is in part caused by the oxidative stress of the procedure. In contrast, the platelet dysfunction during inhaled NO therapy is due to platelet inhibition by NO with increased cyclic GMP levels in platelets. Both studies support the importance of close monitoring of not only platelet number but also platelet function in critically ill neonates treated with these novel interventions. Moreover, platelet activation and dysfunction during ECMO was associated with the release of MMP-2, which may have additional effect on the platelet dysfunction during ECMO.

FUTURE DIRECTIONS

Although the cardioprotective effect of GSH was independent of total GSH concentration, its vasodilator and cardioprotective effects were enhanced with the use of membrane permeable analogs such as GSH mono-ethyl ester. As suggested by the protection against $ONOO^-$-induced aconitase inhibition offered by different thiols, further studies are needed to investigate the cardiac effects of sulfhydryl group-containing compounds (thiols) with greater bioavailability and thus better effectiveness.

The acute role of MMP-2 in the development of myocardial stunning injury is important and also compliments the role of MMP-2 in the regulation of platelet aggregation in vivo under pathological conditions such as ischemia-reperfusion injury which is associated with platelet and leukocyte activation.[1] Thus studying the role of MMP-2 (both the release and activation) using MMP-2 inhibitors or mimetics in myocardial stunning injury will be pursued. Use of N^G-monomethyl-L-arginine and superoxide dismutase may help to test the hypothesis that $ONOO^-$ activates pro MMP-2 isolated hearts subjected to ischemia-reperfusion. Given the limited improvement of cardiac function by *o*-phenanthroline and MMP-2 antibody which could be related to the cardiac depressive action and the bioavailability to the cell, respectively, there is a need to develop effective MMP-2 inhibitors with better selectivity and bioavailability.

In therapeutic interventions involving extracorporeal circulation such as ECMO, cardiopulmonary bypass and hemodialysis, various adjunctive therapies such as the use of inhibitors of prostaglandin synthesis,[14] and supplementation with NO[15]

or NO donors[16] to reduce platelet activation have been tried. Controlled reoxygenation with partial arterial oxygen pressures $\leq$ 100 mmHg has been shown to improve cardiac function following bypass surgery of cyanotic heart disease in newborn piglets,[17] whereas the effect on platelet function was not studied. Being a novel mediator of platelet aggregation, there may be a role for MMP-2 inhibition therapy to attenuate platelet dysfunction during ECMO. This novel therapy may be important as an alternative or complimentary therapy to prevent platelet activation during extracorporeal circulation. Furthermore, studies on the tissue inhibitors of MMP-2 are required to understand their interaction with MMP-2 and thus the pathological conditions in critically ill patients.

Based on the assumption that MMP-2 is an important player in the regulation of tissue remodeling, thrombo-hemostatic balance and cardiac function, MMP-2 may play a role in the pathogenesis of different neonatal diseases including bronchopulmonary dysplasia and intracranial hemorrhage. Dysregulation in the production and activation of MMP-2 can result in pathological fibrotic changes, cardiovascular and platelet dysfunction (hypotension and hemostatic imbalance, respectively). I will study the use of plasma MMP-2 as a clinical marker for the development of bronchopulmonary dysplasia and intracranial hemorrhage in high-risk neonates. This can provide additional information in the management of these sick neonates and help us to understand the pathogenesis of these neonatal diseases.

The inhibitory effect of inhaled NO on platelet function has been understated in the current use of inhaled NO for the treatment of critically ill neonates. Inhalation of NO improves systemic oxygenation with minimal risk of side effects reported.[12]

The inhibited platelet function, with possibly increased risks for hemorrhagic complications especially in premature neonates, is of particular concern and warrants caution. Careful patient selection criteria such as prematurity, evidence of disseminated intravascular coagulopathy and clinical bleeding complications need to be established. Also, clinical monitoring of the hemostatic balance is required during the administration of this novel experimental therapy to critically ill neonates. An antagonist to the inhibitory effect of NO with platelet selectivity (such as platelet specific antibody-linked drugs) should be developed. On the other hand, the platelet inhibitory effect of inhaled NO may have a role in the prevention or attenuation of platelet activation during the use of an extracorporeal circulation. Further investigations are required to explore the use of inhaled NO such as before and during ECMO to induce simultaneous pulmonary vasodilation and platelet deactivation resulting in improvements in both oxygenation and platelet function.

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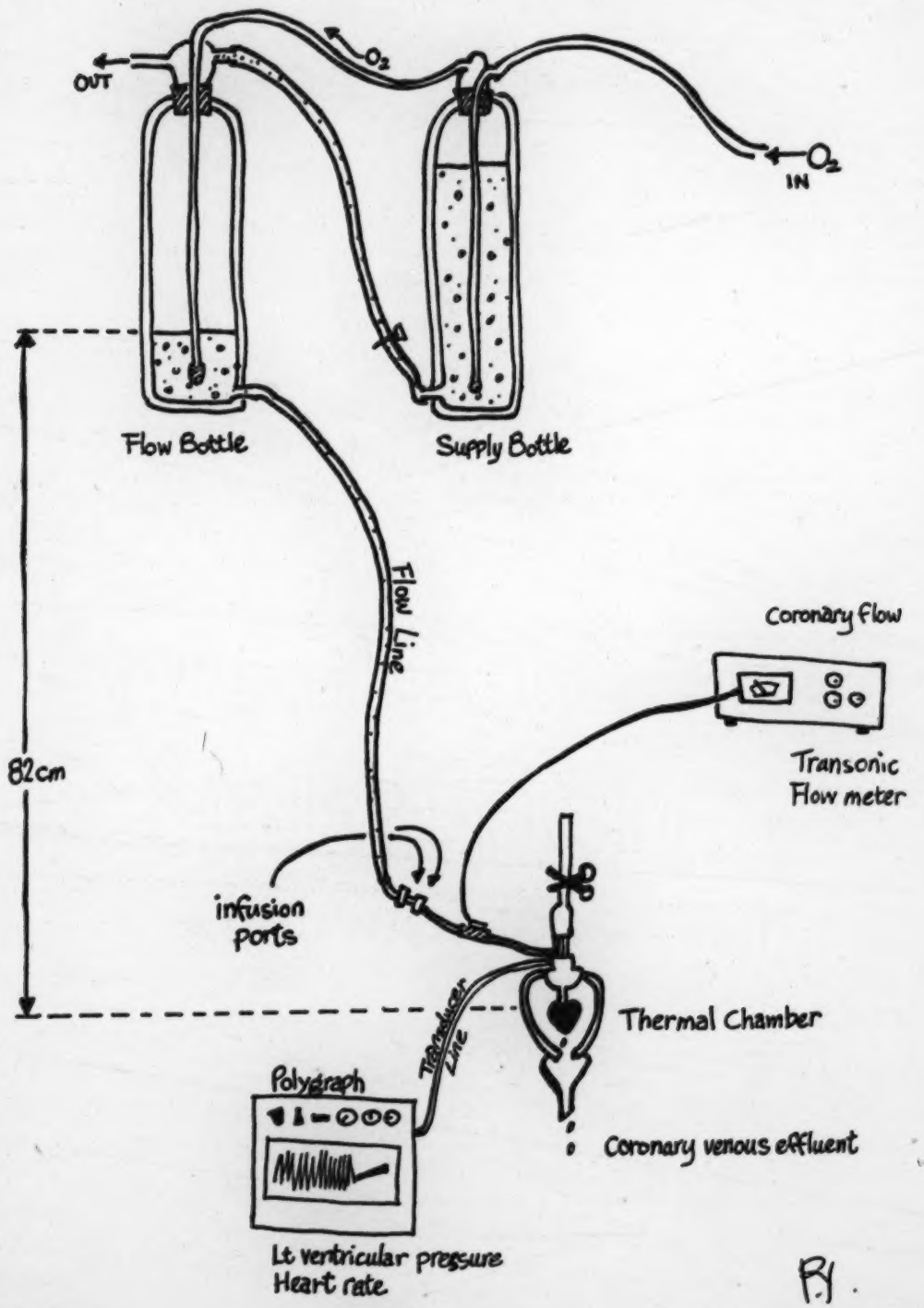
APPENDIX I

SCHEMATIC OF THE ISOLATED RAT HEART SET-UP

The Isolated Langendorff Rat Heart at Constant Pressure Perfusion



APPENDIX II

NITRIC OXIDE AND PLATELET FUNCTION: IMPLICATIONS FOR NEONATOLOGY

A version of this chapter has been published. Cheung P-Y, Salas E, Schulz R, Radomski MW. (1997) Sem Perinatol 21:409-417.

ABSTRACT

Nitric oxide (NO) is a mediator that modulates vessel wall tone and hemostatic-thrombotic balance. Platelet function is regulated by NO generated from platelets, endothelial cells and leukocytes. Nitric oxide has been shown to inhibit platelet adhesion, aggregation, and stimulate disaggregation of preformed platelet aggregates. Many of the effects of NO are mediated by its stimulation of guanylate cyclase and the formation of cyclic GMP and its subsequent transduction mechanism. In vivo, NO is likely to interact with prostacyclin, metabolites of ecto-nucleotidase and lipoxygenase to modulate platelet function in a synergistic manner. An imbalance of NO production (deficiency or overproduction) has been implicated in the pathogenesis of various vascular disorders including thrombosis, atherosclerosis, septicemia and ischemia-reperfusion injury. It is likely that some of detrimental effects of NO are mediated through its reaction with superoxide anion to form the potent oxidant, peroxynitrite. Nitric oxide gas and NO donors are used for the pharmacological treatment of various vascular disorders. Since inhaled NO has been documented to improve systemic oxygenation and reduce the need for extracorporeal membrane oxygenation, it has been widely used in neonates with severe hypoxemia. An inhibition of platelet function, resulting in a prolonged bleeding time, has been demonstrated in adults receiving inhaled NO. Since bleeding complications may occur in high-risk infants, it is important to evaluate the effect of inhaled NO on platelet function and its correlation with clinical consequences such as intracranial hemorrhage. For these reasons, hemostasis should be carefully monitored during the administration of inhaled NO to critically ill neonates.

INTRODUCTION

Nitric oxide (NO) has been shown to be an important physiologic and regulatory mediator that modulates vessel wall tone and hemostatic-thrombotic balance via the stimulation of the soluble guanylate cyclase.[1,2] Changes in its generation or metabolism contribute to the pathogenesis of vascular and thrombotic disorders. Nitric oxide also accounts for the pharmacological activity of various NO-donating drugs including organic nitrates.[3]

Inhalation of NO gas has been used as one of the therapeutic approaches to the treatment of lung disorders associated with hypoxia and pulmonary vasoconstriction.[4] Inhaled NO has also been used to treat critically ill neonates with severe hypoxemia.[5] This treatment can increase systemic oxygenation by improving ventilation-perfusion matching with or without lowering of pulmonary vascular resistance.[6] One of the main advantages of the use of inhaled NO is its short biological half-life. It has been postulated that inhaled NO should act only locally at the level of vessel wall and will not exert significant systemic effects due to its rapid inactivation by red cell oxyhemoglobin.[4] However, platelets, in contrast to erythrocytes, are in close contact with the pulmonary vascular endothelium and therefore platelet function could also be affected by inhaled NO. In this chapter, I will review the data related to the effects of endogenous and pharmacologically administered NO on platelet function and vessel wall homeostasis relevant to neonatology.

Endogenous Nitric Oxide: Actions and Signal Transduction Mechanism

Nitric oxide is generated from a two-step hydroxylation-oxidation of L-arginine catalyzed by NO synthase enzymes (NOS).[7] There are at least three isoforms of NOS which have been named on the basis of the cell types they were originally discovered in: nNOS from neurons, eNOS from endothelial cells and iNOS, an isoform expressed in macrophages and many other cell types in response to immunological stimulation.[8,9] Blood platelets generate NO via a NOS-dependent pathway.[10] Indeed, L-arginine, the substrate for NOS, inhibits platelet aggregation.[10] Conversely, the inhibition of NOS potentiates aggregation induced by different aggregating agents in vitro.[11] The activity of Ca^{2+}-dependent NOS was found in the cytosolic fraction obtained from human platelet homogenates.[10] It appears that the enzyme shows a biochemical profile similar to nNOS and eNOS: i.e. it is Ca^{2+}-, calmodulin-, NADPH-, FAD-, tetrahydrobiopterin- and L-arginine-dependent.[10,12] The activity of Ca^{2+}-independent NOS (iNOS) has been found in cytokine-treated platelets[13] and platelets from healthy volunteers.[14] The platelet iNOS may be both of megakaryocyte and platelet origin as there is evidence for the presence of platelet-specific mRNA and protein synthesis in platelets.[15]

A substantial part of NO-operated transduction mechanism depends upon its stimulation of the soluble guanylate cyclase and increased cyclic GMP levels.[16] Cyclic GMP activates a cyclic GMP-dependent protein kinase and phosphorylates various target proteins,[17] including a vasodilator stimulated phosphoprotein (VASP, molecular weight 46-50 kDa).[18] This protein is associated with the platelet cytoskeleton and contributes to the regulation of platelet adhesion receptors, including IIb/IIIa and P-

selectin, and the inhibition of platelet adhesion.[19,20] Cyclic GMP also decreases basal and stimulated concentrations of intracellular Ca^{2+} levels by modulating the activity of Ca^{2+} handling systems in platelets.[21]

Nitric oxide may also regulate platelet function via cyclic GMP-independent effects. Nitric oxide may interact the arachidonic acid cyclooxygenase[22] and 12-lipoxygenase enzymes,[23] some enzymes of the mitochondrial respiratory chain,[24] glyceraldehyde-3-phosphate dehydrogenase[25] and intracellular thiols such as glutathione.[26] The significance of cyclic GMP-independent effects of NO on platelet function remains to be established.

Role of NO in the Physiological Regulation of Platelet Function

When a blood vessel is damaged and the endothelium is disrupted, platelets are recruited from the circulating blood to form an occlusive plug. *Platelet adhesion* is initiated by the exposure of adhesive portions of vessel wall (subendothelium, media, adventia) that are normally concealed from the blood by an intact endothelial monolayer. The interactions of vascular adhesion elements with specific receptors (integrins or other) on platelets allow the anchorage of platelets to components of the subendothelial matrix. Soluble factors circulating in plasma, such as epinephrine, ADP, vasopressin, serotonin and thrombin, amplify the platelet membrane signals initiated by adhesion to the subendothelial matrix leading to *platelet aggregation*. Aggregation involves the activation of lipid-metabolizing enzymes, such as phospholipases A_2 and C, and results in a dramatic reorganization of platelet cytoskeleton and the release of platelet constitutents to the extracellular compartment. Platelet aggregation is reinforced by ADP, thromboxane A_2 and a metalloproteinase enzyme, gelatinase A.[27] Platelet aggregation culminates with the stabilization of loose bridges between adjacent platelets by fibrinogen and von Willebrand Factor due to conformational changes in the integrin receptors IIb/IIIa. Furthermore, the translocation of P-selectin to the platelet surface membrane causes the formation of platelet-leukocyte aggregates.

Nitric oxide is involved in the modulation of all stages of platelet activation by inhibiting adhesion, aggregation, and stimulating disaggregation of platelet aggregates both in vitro,[28] and in vivo[29] (Figure 1). Nitric oxide can regulate platelet function in both *autocrine* (NO generated inside platelets) and *paracrine* (NO generated outside platelets) manners. The autocrine regulation is based on the platelet activation-

dependent increase in the activity of eNOS that offsets platelet activation via a soluble guanylate cyclase and cyclic GMP-dependent mechanism.[10,11,30]

The paracrine regulation of platelet function is based on the continuous generation, release, and diffusion of NO from endothelial cells and leukocytes to the platelet microenvironment. It is likely that pulsatile flow and changes in shear stress are responsible for the constant generation and release of NO by vascular endothelial cells.[31] Interestingly, pulsatile blood flow and the shear rate are also major determinants of platelet behavior in vivo.[32] Under physiological conditions, in the whole blood, while the more numerous and larger erythrocytes occupy the axial stream, platelets assume the position close to the endothelial cells. This rheological arrangement allows an efficient transfer of NO from the endothelium to platelets.

A number of animal and human studies support an important role of NO in the regulation of platelet function in vivo. These studies have demonstrated that: (1) basal or stimulated NO production results in inhibition of platelet aggregation induced by some aggregating agents or endothelial injury[33] and also increases bleeding time,[34] (2) luminal release of NO from human vasculature increases intraplatelet cyclic GMP levels,[35] and (3) the administration of the NOS inhibitor N^G-monomethyl-L-arginine reversibly increases platelet aggregation and granule release in healthy volunteers.[36] The contribution of NO released from neutrophils to the regulation of platelet function in vivo remains to be established.[37] The expression of P-selectin and formation of platelet-leukocyte aggregates may be regulated by NO released from leukocytes.[38]

Metabolites of both cyclooxygenase and lipoxygenase (particularly prostacyclin and 13-hydroxyoctadecadienoic acid, respectively), as well as ecto-nucleotidase show

important synergistic actions with NO to regulate platelet function. Prostacyclin, a potent inhibitor of platelet aggregation that acts by stimulating the adenylate cyclase system, is a weak inhibitor of platelet adhesion.[39] The resultant cyclic AMP levels down-regulate the transduction mechanism for platelet activation, including inhibition of intracellular Ca^{2+} release and activation of platelet receptors.[40] The ecto-nucleotidase metabolizes ADP to AMP and adenosine, which in turn increases intracellular cyclic AMP levels, and thereby limits platelet recruitment and reactivity.[41] 13-Hydroxyoctadecadienoic acid is synthesized from linoleic acid by lipoxygenase in endothelial cells. This fatty acid regulates platelet adhesion by modulating integrin receptor expression.[42]

Role of NO in Platelet Function under Pathological Conditions

The vasodilator and platelet-regulatory functions of endothelium are impaired with altered bioactivity of NO in various vascular diseases, including essential hypertension,[43] diabetes[44] and coronary artery disease.[45] Studies have shown that oxidized or low-density lipoproteins decrease the bioactivity of NO by various mechanisms including: (1) inhibition of NOS activity, (2) direct inactivation of NO, (3) changes in NO metabolism, and (4) inhibition of L-arginine uptake into platelets.[45,46,47,48,49] In contrast, high-density lipoproteins prevent thrombosis by inhibiting platelet function that is associated with increased NOS activity.[50] Interestingly, during atherogenesis, there is an increased expression of iNOS[51] and this may result in cellular toxicity. The mechanism of cytotoxic effects of NO dreived from iNOS may be indirect and involve its reaction with superoxide anion to form peroxynitrite ($ONOO^-$).[52,53] Indeed, $ONOO^-$ is a potent and highly reactive oxidant that causes endothelial injury.[54] Peroxynitrite stimulates aggregation of human platelets[55] and decreases the relaxation response of endothelium-dependent vasodilators.[56] These responses are reversed or attenuated by exogenous NO or prostacyclin[55,56] or in the presence of thiols and glucose that react with $ONOO^-$ to form NO donors.[56,57] Thus, the net effect (cytoprotective or cytodestructive) of inducible NO is likely to be dependent on the microenvironment where this molecule is generated, reacts and acts.[58]

Sepsis syndrome is common in neonates. The breakdown products of NO in vivo, serum nitrite and nitrate, are increased in children with sepsis, especially those with hypotension.[59] It has been shown that iNOS is expressed during septicemia and septic shock.[60] Following the invasion of bacteria and exposure of cells to bacterial

exotoxin or endotoxin resulting in the production of pro-inflammatory cytokines, iNOS is induced and large amounts of NO are generated. This results in complex effects on the hemostatic-thrombotic balance and microvascular perfusion. The inducible NO triggers a progressive loss in vascular reactivity, profound hypotension and endothelial cell damage.[60,61] These detrimental effects of inducible NO in sepsis can be antagonized by inhibiting NOS with N^G-monomethyl-L-arginine.[62] However, since the originally available NOS inhibitors lack selectivity and inhibit all isoforms of NOS, caution should be exercised during the administration of these compounds in sepsis. Indeed, non-selective NOS inhibitors can potentiate cytokine-stimulated platelet adhesion to endothelial cells,[63] precipitate renal glomerular thrombosis[64] and exacerbate sepsis-induced renal hypoperfusion.[65] Thus, generation of appropriate amount of NO is crucial to maintain the integrity of the microvasculature during sepsis.[66] The effect of $ONOO^-$, which is likely to be generated during sepsis,[67] on hemostatic-thrombotic balance remains to be elucidated. Unraveling the roles of nitrogen- and oxygen-derived radicals species may help to explain some clinical symptoms of sepsis, such as thrombocytopenic-thrombotic phenomenon, myocardial dysfunction and hyporeactivity with poor tissue perfusion.[68]

L-arginine-NO pathway plays an important role in ischemia-reperfusion injury[69] and hypoxia-reoxygenation injury.[70] In this pathology, NO is also considered as a double-edged sword exerting both beneficial and detrimental effects. On one hand, NO is cytoprotective through vasodilator, platelet- and leukocyte- inhibitory effects. On the other hand, NO can be cytodestructive following its conversion to $ONOO^-$. Indeed, $ONOO^-$ formation is increased in the early stage of reperfusion/reoxygenation in the

heart.[69] This burst of $ONOO^-$ causes impaired recovery of myocardial function,[69] endothelial[56] and platelet dysfunction.[55] Yasmin et al have shown an improvement of myocardial mechanical function during reperfusion in isolated rat hearts by limiting the amount of $ONOO^-$ production using a NOS inhibitor[69,71] and a cell-permeable superoxide dismutase mimetic.[72]

Inhaled NO and platelet function in critically ill neonates

Inhaled NO therapy has been increasingly used in neonates following reports on improvement in oxygenation in term neonates with severe hypoxemia,[5,6] and also in premature infants with suspected hypoplastic lungs and intractable hypoxemia unresponsive to aggressive ventilatory support and surfactant treatment.[73] Studies by Finer et al have showed that the beneficial effects of inhaled NO in critically ill neonates may depend, in part, on the improved ventilation-perfusion matching.[6,74] Recently, two double-blinded, randomized controlled trials have documented the efficacy of inhaled NO in improving systemic oxygenation in critically ill neonates with severe hypoxemia with or without pulmonary hypertension.[75,76] Despite the significant reduction in the requirement for extracorporeal membrane oxygenation in the group of neonates that underwent NO therapy, mortality in these neonates was similar to that of untreated neonates. Interestingly, both studies indicated a low incidence of adverse effects such as methemoglobinemia and increase in nitrogen dioxide concentrations.

Several reports have been published regarding the effect of inhaled NO on platelet aggregation (Figure 2). Nitric oxide gas is a potent inhibitor of platelet adhesion and aggregation in vitro.[77] However its antiplatelet activity is limited by short half-life (seconds) in biological milieu.[28] Hogman et al reported a prolongation of the bleeding time in normal adults who received inhaled NO,[78] and also demonstrated a prolonged bleeding time in rabbits receiving as little as 3 ppm of inhaled NO.[79] Recently, Samama et al have shown a decrease in collagen-induced platelet aggregation in a study of 6 critically ill adults with acute respiratory distress syndrome treated with inhaled NO.[80]

Interestingly, inhalation of NO by healthy volunteers[78] and experimental animals[79] resulted in a longer-lasting (approximately 30 min) inhibition of platelet hemostasis as detected by prolongation of bleeding time. This phenomenon may be explained either by accumulation and subsequent slow release of NO from the lipid part of cell membrane or formation of endogenous NO donors capable of long-lasting release of NO such as *S*-nitrosothiols. The formation of endogenous stores of NO following inhalation of this gas is supported by a recent report of a second peak of serum nitrate and nitrite concentrations at 3 hr following the cessation of inhaled NO in human volunteers.[81] Poor oxygenation, alterations of antioxidant stores[82] and an immature pathway of NO metabolism in neonates may all contribute to this long-lasting biological effect of inhaled NO on platelet function.

Bleeding is an important complication that affects mortality and long-term neurodevelopmental outcome in critically ill neonates. Intracranial hemorrhage is a common finding in premature infants, and it has been reported in approximately 40 % of infants with birth weight less than 1500 g.[83] Platelet dysfunction contributes to the precipitation or aggravation of intracranial hemorrhage.[84] Hemorrhage and deranged platelet aggregation have been shown in neonates with septicemia.[85,86] In a review of the Extracorporeal Life Support Organization Registry, Horwitz et al found that culture-positive, septic neonates who require extracorporeal membrane oxygenation appear to be at an increased risk for intracranial hemorrhage and death.[87] Intracranial hemorrhage appears to be the primary factor affecting survival in these patients. In these high-risk infants whose platelet function is already compromised by the existing hypoxemia and acidemia,[88,89] the risk of bleeding during inhaled NO therapy may be

exaggerated and lead to the development of intracranial hemorrhage. Inhaled NO therapy could therefore be potentially hazardous for its use in these neonates at risk for intracranial hemorrhage. On the other hand, thrombotic complications, such as oxygenator thrombi and hemofilter malfunction occurred more often in those neonates who developed sepsis while on extracorporeal membrane oxygenation.[90] Therefore, inhaled NO may also have a beneficial role by inhibiting platelet activation in this condition.[91]

Recently, several groups of investigators have studied the effect of inhaled NO on platelet function in the neonatal population. I have found an inhibition of platelet aggregation stimulated by collagen in neonates receiving 2-20 ppm NO despite normal latelet counts and absence of methemoglobinemia.[92] Moreover, George et al have shown the prolongation of bleeding time without impaired platelet aggregation in neonates receiving inhaled NO at 40 ppm.[93] In contrast, Christou et al have demonstrated that ADP-induced platelet expression of P-selectin was not inhibited by 30 min of 40 ppm inhaled NO in infants with persistent pulmonary hypertension.[94] However, platelet function such as adhesion or aggregation was not examined by these investigators. Thus, the clinical significance of the prolonged bleeding time and inhibited platelet function remains to be established.

Similarly, the relevance of platelet inhibition by inhaled NO to the incidence of bleeding is unknown. Although the incidence of intracranial hemorrhage was not increased in term or near-term neonates treated with inhaled NO in two studies,[75,76] there is increasing concern related to the incidence of intracranial hemorrhage in premature neonates treated with inhaled NO.[95] This is of particular importance in septic neonates

treated with inhaled NO who also have an increased risk of bleeding complications. Therefore, I postulate that there is a need to evaluate the relationship between impaired platelet function and clinically significant bleeding in neonates receiving inhaled NO.

CONCLUSION

Nitric oxide is an important modulator of platelet function acting both in autocrine and paracrine manners that synergises with other regulatory factors such as prostacyclin. Growing evidence for the effect of inhaled NO on platelet function in critically ill neonates justifies a detailed examination of the potential risk of NO-induced platelet inhibition and bleeding using clinical studies. Meantime, caution is advised for the administration of inhaled NO, and careful patient selection criteria should be developed while subjecting these critically ill infants to inhaled NO therapy.

Fig. A2.1 The role of endogenous NO in platelet function.

The eNOS and iNOS are synthesized in the megakaryocyte (MKC) and then passed onto platelets. There is also some evidence for the expression of iNOS by platelets following cytokine stimulation. Both isoforms convert L-arginine (L-arg) to NO and L-citrulline, largely inactive co-product of NOS reaction. Nitric oxide interacts with a number of molecular targets including the soluble guanylate cyclase (GC-S), 12-lipoxygenase (12-LOX), cyclooxygenase (COX), cytochrome C (Cyt. C), superoxide leading to peroxynitrite ($ONOO^-$), thiols leading to *S*-nitrosothiols (R-SNO), and glyceraldehyde-3-phosphate dehydrogenase (GAP-DH). Cyclic GMP, generated following activation of GC-S by NO, inhibits platelet adhesion and aggregation and interacts with cyclic AMP formed in response to prostacyclin (PGI_2) stimulation. Oxyhemoglobin (HbO_2) scavenges NO and converts it to methemoglobin (MetHb).

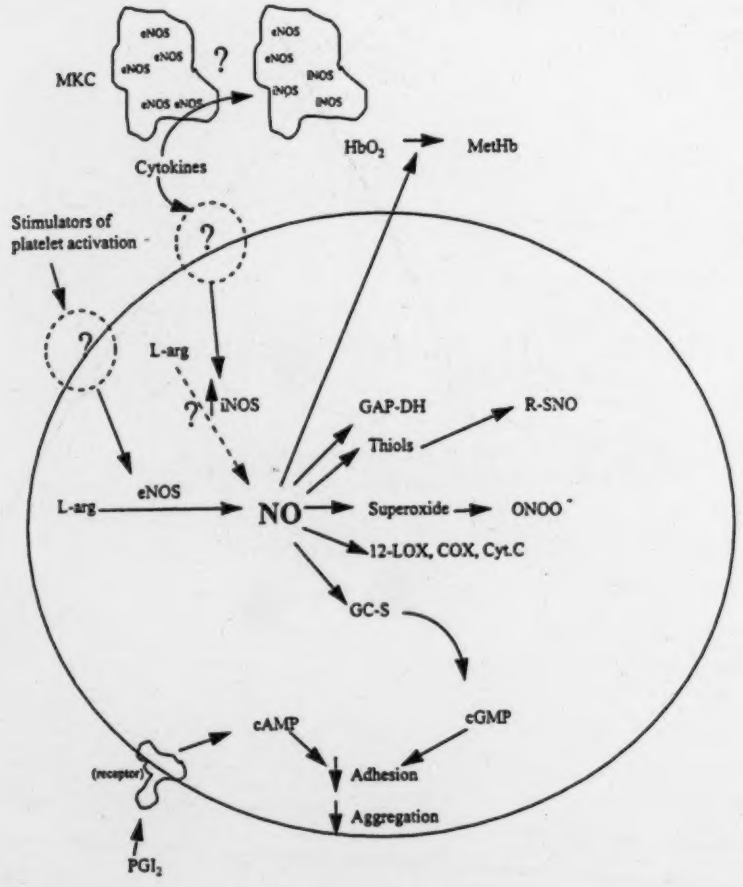
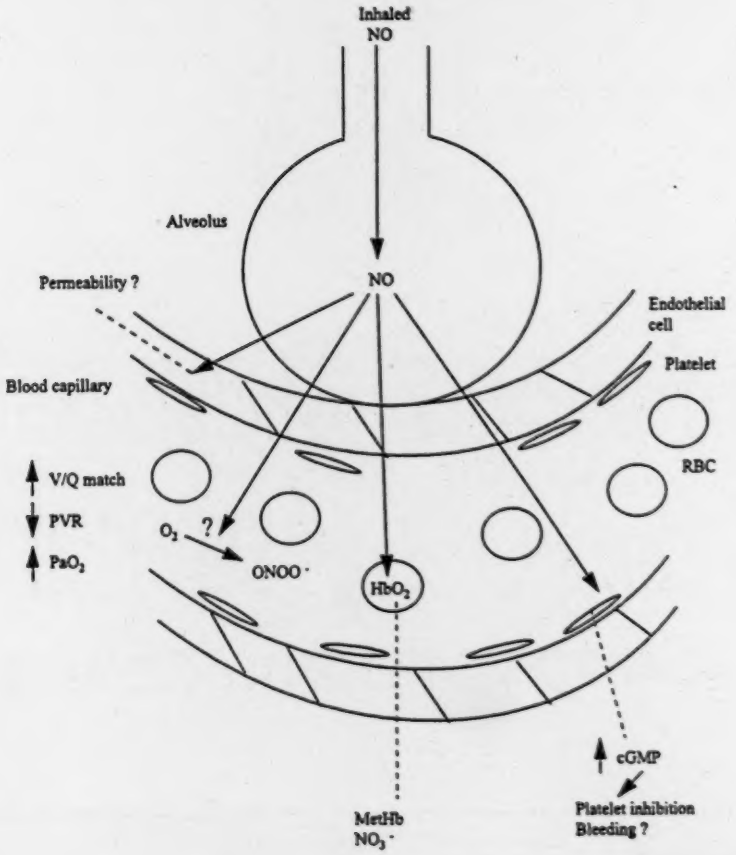


Fig. A2.2 The pharmacological actions of inhaled NO.

Inhaled NO exerts its effects on the pulmonary vasculature improving ventilation-perfusion (V/Q) match, decreasing vascular resistance (PVR) and increasing blood oxygenation (PaO_2). Nitric oxide acts also on platelets leading to inhibition of platelet function and increased likelihood of bleeding. Red blood cells (RBC) containing oxyhemoglobin (HbO_2) inactivate NO by converting it to methemoglobin (MetHb) and nitrate (NO_3^-).



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